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(54) Title: BARLEY WITH ALTERED BRANCHING ENZYME ACTIVITY AND STARCH AND STARCH CONTAINING PRODUCTS WITH AN INCREASED AMYLOSE CONTENT

(57) Abstract: Barley having a reduced level of SBEIIa activity produces grain having a high relative amylose content. The barley might additionally have reduced levels of SBEIIb activity. The barley grain of this invention can be of a non-shrunken phenotype despite a lesion in the amylopectin synthesis pathway.

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BARLEY WITH ALTERED BRANCHING ENZYME ACTIVITY AND STARCH AND STARCH CONTAINING PRODUCTS WITH AN INCREASED AMYLOSE CONTENT

FIELD OF THE INVENTION

This invention relates to a barley plant with a reduced starch branching enzyme IIa (SBEIIa) activity in the endosperm, leading to a kernel starch with an increase in relative amylose content. The invention also relates to grain and starch and food and non-food products obtained therefrom.

BACKGROUND OF THE INVENTION

In cereals, starch makes up approximately 45-65% of the weight of the mature grain. The starch is composed of two types of molecule, amylose and amylopectin. Amylose is an essentially linear molecule composed of α-1,4 linked glucosidic chains, while amylopectin is highly branched with α-1,6 glucosidic bonds linking linear chains.

The synthesis of starch in the endosperm of higher plants is carried out by a suite of enzymes that catalyse four key steps. Firstly, ADP-glucose pyrophosphorylase activates the monomer precursor of starch through the synthesis of ADP-glucose from G-1-P and ATP. Secondly, the activated glucosyl donor, ADP-glucose, is transferred to the non-reducing end of a pre-existing α1-4 linkage by starch synthases. Thirdly, starch branching enzymes introduce branch points through the cleavage of a region of α-1,4 linked glucan followed by transfer of the cleaved chain to an acceptor chain, forming a new α-1,6 linkage. Starch branching enzymes are the only enzymes that can introduce the α-1,6 linkages into α-polyglucans and therefore play an essential role in the formation of amylopectin. Finally, starch debranching enzymes remove some of the branch linkages although the mechanism through which they act is unresolved (Myers *et al.*, 2000).

While it is clear that at least these four activities are required for normal starch granule synthesis in higher plants, multiple isoforms of each of the four activities are found in the endosperm of higher plants and specific roles have been proposed for individual isoforms on the basis of mutational analysis (Wang *et al,* 1998, Buleon *et al.*, 1998) or through the modification of gene expression levels using transgenic approaches (Abel *et al.*, 1996, Jobling *et al.*, 1999, Scwall *et al.*, 2000). However, the precise contributions of each isoform of each activity to starch biosynthesis are still not known, and it is not known

whether these contributions differ markedly between species. In the cereal endosperm, two isoforms of ADP-glucose pyrophosphorylase are present, one form within the amyloplast, and one form in the cytoplasm (Denyer *et al.*, 1996, Thorbjornsen *et al.*, 1996). Each form is composed of two subunit types. The shrunken (*sh2*) and brittle (*bt2*) mutants in maize represent lesions in large and small subunits respectively (Girouz and Hannah, 1994). Four classes of starch synthase are found in the cereal endosperm, an isoform exclusively localised within the starch granule, granule-bound starch synthase (GBSS), two forms that are partitioned between the granule and the soluble fraction (SSI, Li *et al.*, 1999a, SSII, Li *et al.*, 1999b) and a fourth form that is entirely located in the soluble fraction, SSIII (Cao *et al*, 2000, Li *et al.*, 1999b, Li *et al*, 2000). GBSS has been shown to be essential for amylose synthesis (Shure *et al.*, 1983), and mutations in SSII and SSIII have been shown to alter amylopectin structure (Gao *et al*, 1998, Craig *et al.*, 1998). No mutations defining a role for SSI activity have been described.

Three forms of branching enzyme are expressed in the cereal endosperm, branching enzyme I (SBEI), branching enzyme IIa (SBEIIa) and branching enzyme IIb (SBEIIb) (Hedman and Boyer, 1982, Boyer and Preiss, 1978, Mizuno *et al.*, 1992, Sun *et al.*, 1997). In maize and rice, high amylose phenotypes have been shown to result from lesions in the *SBEIIb* gene, also known as the *amylose extender* (*ae*) gene (Boyer and Preiss, 1981, Mizuno *et al.*, 1993; Nishi et al., 2001). In these *SBEIIb* mutants, endosperm starch grains showed an abnormal morphology, amylose content was significantly elevated, the branch frequency of the residual amylopectin was reduced and the proportion of short chains (<DP17, especially DP8-12) was lower. Moreover, the gelatinisation temperature of the starch was increased. In addition, there was a significant pool of material that was defined as "intermediate" between amylose and amylopectin (Boyer *et al.*, 1980, Takeda, *et al.*, 1993b). In contrast, maize plants mutant in the *SBEIIa* gene due a mutator (Mu) insertional element and consequently lacking in SBEIIa protein expression were indistinguishable from wild-type plants in the branching of endosperm starch (Blauth *et al.*, 2001), although they were altered in leaf starch. Similarly, rice plants deficient in SBEIIa activity exhibited no significant change in the amylopectin chain profile in endosperm (Nakamura. 2002).

In maize, the *dull1* mutation causes decreased starch content and increased amylose levels in endosperm, with the extent of the change depended on the genetic background, and increased degree of branching in the remaining amylopectin (Shannon and Garwood, 1984). The gene corresponding to the mutation was identified and isolated by a transposon-tagging

strategy using the transposon mutator (Mu) and shown to encode the enzyme designated starch synthase II (SSII) (Gao et al., 1998). The enzyme is now recognized as a member of the SSIII family in cereals. Mutant endosperm had reduced levels of SBEIIa activity associated with the *dull1* mutation. No corresponding mutation has been reported in other cereals. It is not known if these findings are relevant to other cereals, for example barley.

WO94/09144 suggests the use of sense and antisense genes to alter the natural ratios of starch synthase (SS) and SBE in maize. However, no data are presented to substantiate the proposed molecular strategies and there is no suggestion of specifically reducing the activity of SBEIIa.

In potato, down regulation of *SBEI* alone causes minimal affects on starch structure (Filpse *et al.*, 1996), although further work identified some qualitative changes (Safford et al., 1998). However, in potato the down regulation of *SBEII* and *SBEI* in combination increased the relative amylose content much more than the down-regulation of *SBEII* alone (Schwall *et al.*, 2000).

Two types of debranching enzymes are present in higher plants and are defined on the basis of their substrate specificities, isoamylase type debranching enzymes, and pullulanase type debranching enzymes (Myers *et al.*, 2000). Sugary-1 mutations in maize and rice are associated with deficiency of both debranching enzymes (James *et al.*, 1995, Kubo *et al.*, 1999) however the causal mutation maps to the same location as the isoamylase-type debranching enzyme gene. In the *Chlamydomonas* sta-7 mutant (Mouille *et al.*, 1996), the analog of the maize *sugary-1* mutation, isoamylase activity alone is down regulated. Starch biosynthesis genes that have been cloned from cereals are listed in Table 1.

Starch is widely used in the food, paper and chemical industries. The physical structure of starch can have an important impact on the nutritional and handling properties of starch for food or non-food or industrial products. Certain characteristics can be taken as an indication of starch structure including the distribution of amylopectin chain length, the degree of crystallinity and the presence of forms of crystallinity such as the V-complex form of starch crystallinity. Amylopectin chain length may be an indicator of altered crystallinity and altered gelatinisation and is also thought to have a correlation with reduced retrogradation of amylopectin. Additionally, varied amylopectin chain length distribution is thought to reflect organoleptic properties of food in which the starch is included in

significant amounts. Reduced crystallinity of a starch may also be indicative of a reduced gelatinisation temperature of starch and is thought to be associated with enhanced organoleptic properties.

The relatively high gelatinisation temperature of most high amylose starches is a disadvantage for certain food applications. Gelatinisation temperature is reflective of the comminution energy required to process such foods. Higher temperatures are normally required to process grain or flour to manufacture foods from such grains or starches. Therefore, products having high amylose starches are generally more expensive. In addition, consumers may need to use longer times and higher temperatures to prepare the manufactured foods or to make foods from flour having high amylose starches. High amylose starches having reduced or normal gelatinisation temperatures would be advantageous in many food applications.

Starch composition, in particular the form called resistant starch, has important implications for bowel health, in particular health of the large bowel. Accordingly, high amylose starches have been developed in certain grains such as maize for use in foods as a means of promoting bowel health. The beneficial effects of resistant starch result from the provision of a nutrient to the large bowel wherein the intestinal microflora are given an energy source which is fermented to form *inter alia* short chain fatty acids. These short chain fatty acids provide nutrients for the colonocytes, enhance the uptake of certain nutrients across the large bowel and promote physiological activity of the colon. Generally if resistant starches or other dietary fibre is not provided the colon is metabolically relatively inactive.

Another nutritional component of the grains and in particular of barley is β-glucan. β-glucan consists of glucose units bonded by β (1-4) and/or β (1-3) glycosidic linkages and are not degraded by human digestive enzymes, making them suitable as a source of dietary fibre. β-glucans can be partially digested by endogenous colonic bacteria which fermentation process gives rise to short chain fatty acids (predominantly acetate, propionate and butyrate) which are beneficial to mucosal cells lining the intestine and colon (Sakata and Engelhard, 1983). Ingestion of β-glucan also has the effect of increasing bile acid excretion leading to a reduction in total serum cholesterol and low density lipoproteins (LDL) with a lowering of the risk of coronary disease. Similarly β-glucan acts by attenuating excursions in postprandial blood glucose concentration. It is thought that these effects may also be based on the increase of viscosity in the contents of the stomach and intestines.

Whilst modified starches or β glucans, for example, can be utilised in foods that provide functionality not normally afforded by unmodified sources, such processing has a tendency to either alter other components of value or carry the perception of being undesirable due to processes involved in modification. Therefore it is preferable to provide sources of constituents that can be used in unmodified form in foods.

Barley (*Hordeum vulgare*) is the fourth largest cereal grain crop produced worldwide and is relatively underutilized in terms of human consumption aside from its use to produce alcoholic beverage. On average, barley grain contains about 64% starch, 11% protein and 5% β-glucan (normally 3-6%). The remaining 20% includes moisture, fiber and other minor components.

Known variation in barley starch structure is limited relative to the variation available in maize. Mutants in *SBEIIb*, corresponding to the amylose-extender phenotypes in maize or rice, have not been characterized in barley. The phenotype conferred by *SBEIIa* or *SBEIIb* mutations in barley is unknown. The most highly characterised mutations are waxy and a high amylose mutation identified as AC38. High Amylose Glacier (AC38) has relatively modest increases in amylose content to a maximum of about 45% of total starch. Double mutants with a waxy phenotype have also been constructed and analysed (Schondelmaier *et al.*, 1992; Fujita *et al*, 1999).

Other mutants of barley having high amylose starch contents have been identified. Chemically induced mutants in the *SSIIa* gene had higher levels of amylose in kernel starch, to about 65-70% (WO 02/37955 A1). The mutants M292 and M342 also showed substantially reduced average grain weight as a consequence of reduced starch synthesis, from a mean weight of about 51 mg for the parent line Himalaya to 32 and 35 mg for M292 and M342, respectively. Although the mutants retained the length and width of the wild-type grain, they were flattened from 2.8 mm average thickness for Himalaya to 1.6-1.8 mm thickness and had an essentially unfilled central region, which resulted in poorer milling characteristics. The ratio of grain length (L) to thickness (T) was found to be a useful diagnostic parameter for the mutant alleles, with mutants and wild-type seeds having an L:T ratio of >3.5 and <3.5 respectively. The starch content of the mutant lines was reduced from 49.0% for Himalaya to 17.7 and 21.9% for M292 and M342, respectively. It was shown that while there was a decrease in amylose content per grain from 6.2 mg per caryopsis to

4.0 and 4.8 mg in M292 and M342, respectively, there was a dramatic reduction in amylopectin content per caryopsis from 18.7 in Himalaya to 1.6 and 2.9 mg in the mutants. This showed that the high relative amylose level was a result of decreased amylopectin production. Grain β-glucan levels were increased in the mutants to above 10%. The starch showed reduced gelatinisation temperatures. The *SSIIa* mutants had an altered distribution of SBEIIa and SBEIIb activities between the starch granule and soluble fractions of the endosperm, however, they were essentially unaltered in the level of these activities in the endosperm as a whole (WO 02/37955; Morell *et al.*, 2003).

Whilst elevated amylose starches of these types are useful, a barley starch with higher amylose contents is preferred, in particular if associated with improved starch synthesis and other characteristics, for example a reduced need for post-harvest modification. Such starch products are also relatively resistant to digestion and bring a greater health benefit.

GENERAL

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description. The references mentioned herein are hereby incorporated by reference in their entirety. Reference herein to prior art, including any one or more prior art documents, is not to be taken as an acknowledgment, or suggestion, that

said prior art is common general knowledge in Australia or forms a part of the common general knowledge in Australia.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents Thymidine.

SUMMARY OF THE INVENTION

In a first aspect the invention might be said to reside in grain obtained from a barley plant, the barley plant having a reduced level of SBEIIa enzyme activity in the endosperm, starch of said grain having a relative amylose content of at least 40% (w/w). The relative amylose content might preferably be higher than 50% or 75%, and preferably the grain is non-shrunken.

In a second aspect the invention might be said to reside in a barley grain comprising starch having a relative amylose content of at least 75% (w/w).

In a third aspect the invention might be said to reside in flour or wholemeal obtained from the grain of the first or second aspects of the invention, or food products incorporating such flour or wholemeal.

In a fourth aspect the invention might be said to reside in starch obtained from grain of a barley plant, the barley plant having a reduced level of SBEIIa enzyme activity in the endosperm, said starch being unmodified and having a relative amylose content of at least 40% (w/w). In a specific form of the fourth aspect the barley plant additionally has a reduced level of SBEIIb enzyme activity in the endosperm.

In a fifth aspect the invention might be said to reside in a composition comprising the starch according to the fourth aspect of the invention and another food ingredient or water.

In sixth aspect the invention might be said to reside in a composition comprising starch granules of barley endosperm and another food ingredient or water, wherein the starch of the starch granules comprises at least 75% (w/w) amylose.

In a seventh aspect the invention might be said to reside in a barley plant having a reduced level of SBEIIa enzyme activity, wherein starch in grain of the barley plant has a relative amylose content of at least 40% (w/w) or preferably at least 50% or at least 75%.

In an eight aspect the invention might be said to reside in a method of producing a barley plant with a reduced level of SBEIIa enzyme activity in the endosperm, starch of grain of the barley plant having an amylose content of at least 40% (w/w), the method comprising the steps of, a) introducing a genetic variation into a parent barley plant; and b) identifying progeny plants or seed of the parent barley plant that have reduced SBEIIa activity.

In a ninth aspect the invention might be said to reside in a method of producing a barley plant having reduced activity of both SBEIIa and SBEIIb enzyme activities in the endosperm which comprises: a) mutagenising seed from a plant having reduced activity of SBEIIa enzyme activity; or b) mutagenising seed from a plant having reduced activity of SBEIIb enzyme activity; or c) crossing a plant having reduced SEBEIIa enzyme activity with a plant having reduced SBEIIb enzyme activity; and identifying a barley plant having reduced activity of both SBEIIa and SBEIIb.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Nucleotide sequence of the barley SBEIIa cDNA (SEQ ID No. 1).

Figure 2. Nucleotide sequence of the barley SBEIIb cDNA (SEQ ID No. 2).

Figure 3. Sequence of the Starch Branching Enzyme IIa gene (SEQ ID No. 3) (*wSBE II-D1*) from *A. tauschii*, corresponding to the D genome SBEIIa gene of hexaploid wheat (*T. aestivum*).

Figure 4. Partial wheat SBEIIb gene sequence (SEQ ID No. 4) (wbe2b genomic).

Figure 5. Schematic of duplex-RNA constructs. A. The order of the gene elements used were promoter, SBEIIa or SBEIIb gene sequence (exons 1, 2 and 3) in sense orientation, intron (intron 3), SBEIIa or SBEIIb gene sequence (exons 1, 2, 3 and 4) in antisense orientation, and transcription terminator/polyadenylation sequence. B. The transcript of the ds-SBEIIa and ds-SBEIIb genes forms a "hairpin" RNA structure with a double-stranded region formed by hybridization between the sense and antisense sequences. The intron sequence bordered by the G and AG nucleotides is spliced out.

Figure 6. PCR analysis of ds-SBEIIa and ds-SBEIIb transgenic lines of barley. The primer pairs BX17F/AR2bkpnR for SBEIIb and BX17F/AR2akpnR for SBEIIa that amplifies the first and second fragments of respective constructs which included the exons1, 2,3 and intron 3 (sense orientation) were used to identify positive transgenic lines. GP is for the untransformed Golden Promise. The central lane shows molecular size markers.

Figure 7. Southern blot analysis of ds-SBEIIa and ds-SBEIIb transgenic lines of barley. A. Barley ds-SBEIIa positive transgenes as shown by Southern blot hybridization. The expected band size is 1836bp. B. Barley ds-SBEIIb positive transgenes as shown by Southern. The expected band size is 1907bp. GP is Golden Promise (negative control).

Figure 8. Western blot analysis of ds-SBEIIa and ds-SBEIIb transgenic lines of barley. Ten T1 seeds (seeds from T0 plants) of lines IIb 4.3 and IIb4.4 were analysed for SBEIIb expression by Western blot analysis using non-denaturing PAGE and an SBEIIb specific antibody. Lane 1 (+) is for the positive control, variety Glacier.

Figure 9. Western blot analysis of ds-SBEIIa and ds-SBEIIb transgenic lines of barley. T1 seeds (seeds from T0 plants) of line IIa 4.1 were analysed for A. SBEIIa or B. SBEIIb expression by Western blot analysis using non-denaturing PAGE and SBEIIa or SBEIIb specific antibodies. The lanes on both the gels represent the same

seeds. Lane 1 (+) in each panel is for the positive control, variety Glacier.

Figure 10 Western blot analysis of ds-SBEIIa and ds-SBEIIb transgenic lines of barley. T1 seeds (seeds from T0 plants) of line IIb 4.1 were analysed for A. SBEIIb or B. SBEIIa expression by Western blot analysis using non-denaturing PAGE and SBEIIb or SBEIIa specific antibodies. The lanes on both the gels represent the same seeds. The last lane (+) in each panel is for the positive control, variety Glacier.

Figure 11. Starch granule morphology of ds-SBEIIa transgenic barley. Starch granules from single seeds were visualized through light microscopy for both ds-SBEIIa and ds-SBEIIb transgenic seeds. Figure 11A, seed with wild type SBEIIa expression (line IIa4.2.3). Figure 11B, seed which lack SBEIIa expression (line IIa4.2.5). A significant morphological alteration was observed in starch from seeds lacking SBEIIa but not for SBEIIb.

Figure 12. Scanning electron microscopy (SEM) of starch granules. A. wild-type starch granules (line IIa 4.2.3), B. and C. from a ds-SBEIIa transgenic endosperm (line IIa 4.2.5). Starch granules from ds-SBEIIb (SBEIIb inactivated) seed did not appear to be morphologically altered compared to wild-type

DETAILED DESCRIPTION OF THE INVENTION

Alteration of SBEIIa in barley

The invention is based on the finding that a reduction in SBEIIa activity in barley endosperm results in modified starch production, particularly high amylose accumulation in the barley grain. This unexpected result is in contrast to the findings in maize and rice where mutation in SBEIIa did not alter the amylopectin profile (Blauth et al., 20001, Nakamura, 2000). Preferably, there is an alteration in one or more additional starch biosynthetic enzyme activities, and more preferably a reduction in SBEIIb as well as SBEIIa. Preferably also the grain of this barley plant is non-shrunken.

Method of producing a barley plant

In an aspect, the invention provides a method of reducing starch branching enzyme IIa (SBEIIa) activity in the endosperm of barley. The reduction in activity may be by at least 40% or perhaps preferably by at least 50% compared to the level of activity in the endosperm of unmodified (control) barley, more preferably by at least 75%, and even more preferably by at least 90% or 95%. The method may comprise the alteration of the expression of the *SBEIIa* gene of barley, or it may comprise the mutation of the *SBEIIa* gene in barley, whereby the SBEIIa activity in endosperm is reduced.

The method may comprise the step of determining the activity of SBEIIa in barley endosperm, preferably by measuring the level of the protein, for example by immunodetection, or the level of its corresponding mRNA by methods well known in the art, such as Northern blot hybridization analysis or reverse transcription polymerase chain reaction (RT-PCR). The method may further comprise the step of selecting or screening for a barley plant or grain having reduced SBEIIa activity in its endosperm. The selection step may be based on the reduced level of the SBEIIa activity or protein, or it may be based on the phenotype of the grain of the barley plant such as increased amylose content or decreased amylopectin content or a visual phenotype, for example shrunken grain.

SBE activity may be measured by enzyme assay, for example by the phosphorylase stimulation assay (Boyer and Preiss, 1978). This assay measures the stimulation by SBE of the incorporation of glucose 1-phosphate into methanol-insoluble polymer (α-D-glucan) by phosphorylase a. SBE activity can be measured by the iodine stain assay, which measures the decrease in the absorbance of a glucan-polyiodine complex resulting from branching of glucan polymers. SBE activity can also be assayed by the branch linkage assay which measures the generation of reducing ends from reduced amylose as substrate, following isoamylase digestion (Takeda et al., 1993a). Preferably, the activity is measured in the absence of SBEI or SBEIIb activity. Isoforms of SBE show different substrate specificities, for example SBEI exhibits higher activity in branching amylose, while SBEIIa and SBEIIb show higher rates of branching with an amylopectin substrate. The isoforms may also be distinguished on the basis of the length of the glucan chain that is transferred.

In a further aspect, the invention provides a method of reducing the activity of multiple starch biosynthesis enzymatic activities in barley endosperm, wherein one of the activities is SBEIIa. Preferably, the activities of both SBEIIa and SBEIIb are reduced, and even more

preferably SBEI activity is also reduced. Other starch biosynthesis enzymatic activities that may be reduced in combination with SBEIIa are: SSI, SSII, SSIII. Starch debranching enzymes may also be altered, for example the activity of isoamylase or pullulanase. In a further embodiment, the activities of starch biosynthesis enzymatic activities may be altered in the plant in tissues other than endosperm, for example the activity of SBEI or SBEII may be increased in leaves to compensate for some loss of activity caused by a transgene encoding an SBEIIa-inhibitory molecule intended primarily for expression in the endosperm. Alternatively, starch synthesis may be further improved by the overexpression of one or more starch biosynthetic enzymes in combination with a reduction in SBEIIa. Genes encoding such enzymes may be from any of a variety of sources, for example from bacterial or other sources other than barley, and may be modified to alter the catalytic properties, for example alteration of the temperature dependence of the enzymes (WO94/09144).

In a further aspect, the invention provides a method of increasing the level of amylose (as a percentage of starch) in barley grain, comprising the step of reducing the activity of SBEIIa in barley endosperm. The amylose content is preferably at least 50%, more preferably at least 60% and even more preferably at least 65, 75% or 70%. In further preferred embodiments of the invention, the method provides for amylose contents of at least 80% or 90%, as exemplified herein.

The high amylose phenotype may be achieved by partial or full disruption to the expression of the *SBEIIa* gene, or the *SBEIIa* and *SBEIIb* genes. The extent to which the gene is inhibited will in some degree determine the characteristics of the starch made in the barley grain. Any of a range of gel electrophoresis techniques carried out on the proteins extracted from the modified barley endosperm will reveal the nature and extent of modification to the SBEIIa and/or SBEIIb activity. Modification may occur as a reduction in SBEIIa and/or SBEIIb activity, complete abolition of enzyme activity, or an alteration in the distribution of the SBEIIb or other enzymes within the endosperm. To carry out these tests, starch may be extracted from the barley endosperm and the proteins therein analyzed, for example as outlined in Rahman et al, 1995. Techniques well known in the art such as SDS-PAGE and immunoblotting are carried out on the soluble and the starch granule fractions and identify the plants or grain where modifications have occurred to the SBEIIa and/or SBEIIb enzymes.

Barley plants

In a further aspect, the invention provides a barley (*Hordeum vulgare*) plant with a reduced level of SBEIIa activity in the endosperm during at least some of the development of the grain, the barley plant being capable of bearing grain having starch comprising a high relative amylose content. Preferably, the level of SBEIIa is reduced in the endosperm by at least 50%, more preferably by at least 75% and most preferably by at least 90% or 95% compared to the wild-type. The term "wild-type" has its normal meaning in the field of genetics and includes barley cultivars or genotypes which are not modified as taught herein.

The invention also provides progeny plants and grain which have the desired characteristics of the parent.

The invention also encompasses barley plants that have altered SBEIIb or other starch biosynthetic enzyme activities in addition to reduced SBEIIa activity. Plants having reduced SBEIIa and SBEIIb activities may be produced by crossing a plant reduced for SBEIIa with a plant reduced for SBEIIb, or by introducing a transgene encoding a molecule that inhibits expression of both *SBEIIa* and *SBEIIb* genes. The invention also encompasses the mutation(s) in other genetic backgrounds. The original altered (mutant) plants may be crossed with plants containing a more desirable genetic background. After the initial crossing, a suitable number of backcrosses may be carried out to remove the less desirable background. The desired genetic background may include a suitable combination of genes providing commercial yield and other characteristics such as agronomic performance, abiotic stress resistance or hull-less grain. The genetic background might also include other altered starch biosynthesis or modification genes, for example the amylose extender phenotype or the *amo*1 mutation in High Amylose Glacier barley (gene unknown), the waxy mutation (found for example in the Waxiro variety), the mutant gene in the high amylose variety MK6827 (available from the USDA ARS National Small Grain Germplasm Research Facility Aberdeen, Idaho 831290 USA) or the high amylose varieties M292 and M342 (mutation in the *SSIIa* gene) or modifier genes. Additionally it may be desirable to combine other double and triple mutations with combinations of the above lines and in crosses with other barley lines that have a shrunken endosperm where the causal gene is not known.

Grain

The invention also provides barley grain comprising an altered starch compared to wild-type. The altered starch is at least partly a consequence of reduced SBEIIa activity during development of endosperm of the barley grain. The grain comprises increased amylose levels as a percentage of total starch and a reduced amylopectin content compared to wild-type, which has approximately 25% amylose and 75% amylopectin. Preferably, both SBEIIa and SBEIIb activities are reduced during development of the endosperm. Even more preferably, the activity of SBEI is also reduced. The amylose levels, as measured by methods well understood in the art, are preferable at least 50% of the total starch, more preferably at least 60% and even more preferably at least 65%, 70%, 75%, 80% or 90%. Increased amylose levels may be evidenced by abnormal starch granule morphology or loss of birefringence of the granules when observed under a light microscope or other methods. Preferably the amylose level is measured by an iodometric method, which may be spectrophotometric (for example, Morrison and Laignelet, 1983) or by high-performance liquid chromatography (HPLC, for example, Batey and Curtin, 1996).

The grain of the barley plant may have an elevated level of β glucan, which may be associated with increased carbon flow into this polymer rather than into amylopectin synthesis. Alternatively, the grain may have normal levels of β glucan, for example in the range 3.0-6.0% of the mature grain weight. More preferably, the grain comprises both elevated amylose and normal levels of β glucan. Such a combination is unexpected, based on the composition of starch in grain from *SSIIa* mutant barley (WO 02/37955). The grain may comprise starch that has altered gelatinisation temperatures and/or altered swelling characteristics during and following gelatinisation. The grain also, preferably, has a non-shrunken phenotype.

The invention also provides flour or meal produced from the grain. These may be unprocessed or processed, for example by fractionation or bleaching. The invention further provides barley grain useful for food production obtained from a barley plant having an altered level of a SBEIIa activity in the endosperm, starch of said grain having a high amylose content and a reduced amylopectin content. Additionally the invention encompasses grain that has been processed in other ways, so that the grain may have been milled, ground, pearled, kibbled or cracked.

Starch

In another aspect, the invention provides starch obtained from the grain of the barley plant as described above, the plant having a reduced level of SBEIIa activity in the endosperm, the starch having a high amylose content and a reduced amylopectin content. Preferably both SBEIIa and SBEIIb activities are reduced, and more preferably the activity of SBEI is also reduced. In another aspect, the invention provides starch obtained from the grain of the barley plant, comprising at least 50% amylose, preferably at least 60% amylose, and even more preferably at least 65%, 70%, 75%, 80% or 90% amylose. Purified starch may be obtained from grain by a milling process, for example a wet milling process, which involves the separation of the starch from protein, oil and fibre. The initial product of the milling process is a mixture or composition of starch granules, and the invention therefore encompasses such granules. The starch of the granules comprises at least 50%, preferably 70%, 75% or 80% amylose.

The starch may comprise an elevated level of resistant starch, with an altered structure indicated by specific physical characteristics including one or more of the group consisting of physical inaccessibility to digestive enzymes which may be by reason of having a high β-glucan content, altered starch granule morphology, the presence of appreciable starch associated lipid, altered crystallinity, and altered amylopectin chain length distribution. The high amylose content also contributes to the level of resistant starch.

The invention also provides starch from grain of the exemplified barley plant comprising increased amounts of dietary fibre, preferably in combination with the elevated level of resistant starch. This increase is also at least in part a result of the high relative level of amylose.

Methods of reducing gene activity:Transgenes

The activity of SBEIIa and optionally other starch biosynthesis or modification genes are preferably altered by introducing a genetic variation into the plant which might be by means of the introduction of a transgene into the barley plant. A "genetic variation" means any alteration in the genome which, in this context, affects the activity of SBEIIa, and includes mutations such as point mutations, substitutions, inversions, translocations and preferably deletions, as well as introduction of transgenes. A "transgene" as referred to herein has the normal meaning in the art of biotechnology and includes a genetic sequence which has been produced or altered by recombinant DNA or RNA technology and which has been

introduced into the organism or cell of interest. The transgene may include genetic sequence derived from the organism or cell, for example an antisense sequence. The transgene typically includes an exogenous nucleic acid which is not derived from said organism or cell. "Transgenic" refers to the organism or cell containing a transgene. "Non-transgenic" refers to the absence of any transgene in the genome. A transgene is preferably integrated into the genome of the organism or cell, for stable inheritance.

The method of reducing SBEIIa activity may comprise the step of introducing a transgene into a regenerable cell of barley and regenerating a transgenic barley plant from the transformed cell. The branching enzymes involved in synthesis of amylopectin include SBEI, SBEIIa and SBEIIb and the invention encompasses a reduced expression of *SBEIIa* alone or in combination with alteration of *SBEIIb* or *SBEI* expression. Therefore, the transgene(s) may inactivate more than one of these genes. Moreover, the inactivation of SBEIIb and/or SBEI may be direct, in that the transgene (e.g. encoding duplex RNA, antisense, or ribozyme RNA, see below) directly targets the *SBEIIb* or *SBEI* gene expression, or it may indirectly result in the alteration in the expression of *SBEIIb* or *SBEI*. For example, the transgene RNA may target only the *SBEIIa* gene/RNA in terms of sequence identity or basepairing but also result in reduction of SBEIIb or SBEI by altering protein stability or distribution. Additionally forms of the present invention reside in the combination of an altered activity of SBEIIa and an alteration of one or more other amylopectin synthesis enzymes, which enzymes may include SSI, SSII, SSIII, and debranching enzymes such as isoamylase or pullulanase. Expression of any or all of these may be altered by introduction of a transgene.

Several DNA sequences are known for amylopectin synthesis genes in barley, any of which can be the basis for designing transgenes for inactivation of the genes in barley. These include *SBEIIa* (GenBank accession numbers AF064562 and AF064560), *SBEIIb* (GenBank accession numbers AF064563 and AF064561). Homologs of the *SBEI* gene of barley can be isolated by utilising sequences based on DNA sequences from other grains, for example by techniques such as those set out in WO99/14314 to Li *et al.*, for *Triticum*. The *Triticum tauschii* sequence for *SBEI*, which is highly homologous to the wheat D genome *SBEI* gene and has a high degree of similarity to the barley gene, can be found in published Patent specification WO 99/14314 or referenced cited therein, which document is

incorporated herein by reference. The sequence for *SBEI* of wheat can be accessed in the GenBank database under accession number AF076679. Homologues of other amylopectin synthesising genes from wheat or other closely related species can also be used to modify gene expression levels in barley. Such genes or fragments thereof can be obtained by methods well known in the art, including PCR amplification or hybridization to labeled probes.

"Stringent hybridization conditions" as used herein means that hybridization will generally occur if there is at least 90% and preferably at least 95% sequence identity between the probe and the target sequence. Examples of stringent hybridization conditions are overnight incubation in a solution comprising 50% formamide, 5 x SSC (1xSSC = 150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured sheared carrier DNA such as salmon sperm DNA, followed by washing the hybridiazation support in 0.1 x SSC at approximately 65°C. Other hybridization and wash conditions are well known and are exemplified in Sambrook et al, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly chapter11.

The region(s) of the homologues used in preparing the transgene construct should have at least 85% identity to the corresponding barley gene, preferably at least 90% and even more preferably 95-100% identity in the appropriate region. It is also preferred that the transgene specifically target the amylopectin synthesis genes expressed in the endosperm of barley and have less or minimal effect on amylopectin synthesis elsewhere in the plant. This may be achieved by use of suitable regulatory sequences such as endosperm-specific promoters in the transgene.

Antisense

Known genetic engineering or transgenic approaches to altering, in particular specifically reducing, gene activity in plants are well known in the art. These methods of introducing genetic variation into the barley plant include the expression of a suitable antisense molecule that is complementary to the RNA of the target gene and can hybridize with it. Antisense molecules are thought to interfere with the translation or processing or stability of the mRNA of the target gene, thereby inactivating its expression. Methods of devising

antisense sequences are well known in the art and examples of these are can be found in United States Patent No. 5190131, European patent specification 0467349-A1, European patent specification 0223399-A1 and European patent specification 0240208, which are incorporated herein by reference. The use of antisense techniques in plants has been reviewed by Bourque (1995) and Senior (1998). Bourque lists a large number of examples of how antisense sequences have been utilized in plant systems as a method of gene inactivation. She also states that attaining 100% inhibition of any enzyme activity may not be necessary as partial inhibition will more than likely result in measurable change in the system. Senior (1998) states that antisense methods are now a very well established technique for manipulating gene expression.

Antisense molecules for barley *SBEIIa*, *SBEIIb*, *SBEI* or other amylopectin biosynthesis genes can be based on the barley mRNA sequences or based on homologies with DNA or mRNA sequences derived from other species, for example wheat. These antisense sequences may correspond to the structural genes or for sequences that effect control over the gene expression or splicing event. For example, the antisense sequence may correspond to the targeted coding region of the barley *SBEIIa* or other gene, or the 5'-untranslated region (UTR) or the 3'-UTR or combination of these. It may be complementary in part to intron sequences, which may be spliced out during or after transcription, preferably only to exon sequences of the target gene. In view of the generally greater divergence of the UTRs, targeting these regions provides greater specificity of gene inhibition. The length of the antisense sequence should be at least 19 contiguous nucleotides, preferably at least 50 nucleotides, and more preferably at least 100, 200, 500 or 1000 nucleotides. The full-length sequence complementary to the entire gene transcript may be used. The length is most preferably 100-2000 nucleotides. The degree of homology of the antisense sequence to the targeted transcript should be at least 85%, preferably at least 90% and more preferably 95-100%. The antisense RNA molecule may of course comprise unrelated sequences which may function to stabilize the molecule.

Cosuppression

Another molecular biological approach that may be used is co-suppression. The mechanism of co-suppression is not well understood but is thought to involve post-transcriptional gene silencing (PTGS) and in that regard may be very similar to many examples of antisense

suppression. It involves introducing an extra copy of a gene or a fragment thereof into a plant in the sense orientation with respect to a promoter for its expression. The size of the sense fragment, its correspondence to target gene regions, and its degree of homology to the target gene are as for the antisense sequences described above. In some instances the additional copy of the gene sequence interferes with the expression of the target plant gene. Reference is made to Patent specification WO 97/20936 and European patent specification 0465572 for methods of implementing co-suppression approaches.

Double stranded RNA-mediated gene silencing

A further method that might be employed to introduce genetic variation into the barley plant is duplex or double stranded RNA mediated gene silencing. This method also involves PTGS. In this method a DNA is introduced that directs the synthesis of an at least partly double stranded RNA product(s). The DNA therefore comprises both sense and antisense sequences that, when transcribed into RNA, can hybridize to form the double-stranded RNA region. In a preferred embodiment, the sense and antisense sequences are separated by a spacer region that comprises an intron which, when transcribed into RNA, is spliced out. This arrangement has been shown to result in a higher efficiency of gene silencing. The double-stranded region may comprise one or two RNA molecules, transcribed from either one DNA region or two. The presence of the double stranded molecule triggers a response from an endogenous plant system that destroys both the double stranded RNA and also the homologous RNA transcript from the target plant gene, efficiently reducing or eliminating the activity of the target gene. Reference is made to Australian Patent specification 99/292514-A and Patent specification WO 99/53050 for methods of implementing this technique. The length of the sense and antisense sequences that hybridise should each be at least 19 contiguous nucleotides, preferably at least 50 nucleotides, and more preferably at least 100, 200, 500 or 1000 nucleotides. The full-length sequence corresponding to the entire gene transcript may be used. The lengths are most preferably 100-2000 nucleotides. The degree of homology of the sense and antisense sequences to the targeted transcript should be at least 85%, preferably at least 90% and more preferably 95-100%. The RNA molecule may of course comprise unrelated sequences which may function to stabilize the molecule.

Ribozymes

Ribozymes may be used to intorduce the genetic variation responsible for inactivation of the desired gene expression in barley. Ribozymes are RNA molecules with enzymatic or

catalytic function that can cleave other RNA molecules at specific sites defined by one or often two hybridizing sequences. The cleavage of the RNA inactivates the expression of the target gene. The ribozymes may also act as an antisense molecule, which may contribute to the gene inactivation. The ribozymes contain one or more catalytic domains, preferably of the hammerhead or hairpin type, between the hybridizing sequences. Other ribozyme motifs may be used including RNAseP, Group I or II introns, and hepatitis delta virus types. Reference is made to European patent specification 0321201 and US Patent No. 6,221,661. The use of ribozymes to inactivate genes in transgenic plants has been demonstrated, for example by Wegener et al (1994).

Genetic constructs/vectors

The invention also provides isolated nucleic acid molecules including RNA and preferably DNA which encode the gene-inhibiting molecule. Preferably, the nucleic acid molecules encode the antisense, sense (co-suppression), double-stranded RNA or ribozyme molecules targeting the barley *SBEIIa* gene sequence and effective in inactivating its expression in endosperm of barley grain. The invention also provides genetic constructs comprising the isolated nucleic acid molecule, comprising one or more regulatory elements such as promoters, enhancers and transcription termination or polyadenylation sequences. Such elements are well known in the art. The genetic constructs may also comprise intron sequences which aid expression of the transgene in plants, particularly in monocotyledonous plants such as barley. The term "intron" is used in its normal sense as meaning a genetic segment that is transcribed but does not encode protein and which is spliced out of an RNA before translation. Introns may be incorporated in a 5'-UTR or a coding region if the transgene encodes a translated product, or anywhere in the transcribed region if it does not.

The invention further provides vectors, for example plasmid vectors, comprising the genetic constructs. The term "vector" includes an expression vector, being capable of in vitro or in vivo expression, and a transformation vector, capable of being transferred from one cell or organism to another. The vectors comprise sequences that provide for replication in cells, for example in prokaryotic cells such as *E. coli* or *Agrobacterium*. Preferably, the vector is a binary vector comprising a T-DNA sequence, defined by at least one T-DNA border sequence, that can be introduced into barley cells. The invention further provides cells comprising the vectors, for example *Agrobacterium* or barley cells which may be

regenerable cells such as the cells of the scutellum of immature embryos. Alternatively, the cells may be transformed barley cells comprising the transgene.

Promoters/terminators

The transgene or other genetic construct of the invention may include a transcriptional initiation region (promoter) which may provide for regulated or constitutive expression in the endosperm of barley. The promoter may be tissue specific, conferring expression selectively or exclusively in the endosperm. The promoter may be selected from either endosperm-specific (such as High Molecular Weight Glutenin promoter, the wheat SSI promoter, wheat SBEII promoter, wheat GBSS promoter) or promoters not specific for the endosperm (such as ubiquitin promoter or CaMV35S or enhanced 35S promoters). The promoter may be modulated by factors such as temperature, light or stress. Ordinarily, the promoter would be provided 5' of the genetic sequence to be expressed. The construct may also contain other elements that enhance transcription such as the nos 3' or the ocs 3' polyadenylation regions or transcription terminators. The regions of DNA illustrated will be incorporated into vectors containing suitable selectable marker gene sequences and other elements, or into vectors that are co-transformed with vectors containing these sequences.

Transformation methods for barley

Methods for transformation of monocotyledonous plants such as barley for introducing genetic variation into the plant by introduction of an exogenous nucleic acid and for regeneration of plants from protoplasts or immature plant embryos are well known in the art, see for example, Wan and Lemaux (1994), Tingay et al (1997), Canadian Patent Application 2092588 by Nehra, Australian Patent Application No 61781/94 by National Research Council of Canada, Australian Patent No 667939 by Japan Tobacco Inc., International Patent Application PCT/US97/10621 by Monsanto Company, US Patent 5589617, and other methods are set out in Patent specification WO99/14314. Vectors carrying the desired nucleotide sequence or genetic construct and a selectable marker may be introduced into regenerable barley cells of tissue cultured plants or explants, or suitable plant systems such as protoplasts. The selectable marker gene may provide antibiotic or herbicide resistance to the barley cells, or allow the utilization of substrates such as mannose. The selectable marker preferably confers hygromycin resistance to the barley cells. The regenerable barley cells are preferably from the scutellum of immature embryos, mature embryos, callus derived from these, or the meristematic tissue.

The transformed plant may contain a selectable marker gene, or such gene may be removed during or after regeneration, for example by excision of the selectable marker gene out of the genome or by segregation of the selectable marker gene away from the *SBEIIa*-inhibiting transgene.

Plants where the transgene or mutation has been integrated into a chromosome can be screened for by, for example, using a suitable nucleic acid probe specific for the transgene or phenotypic observation. Any of several methods may be employed to determine the presence of a transformed plant. For example, polymerase chain reaction (PCR) may be used to amplify sequences that are unique to the transformed plant, with detection of the amplified products by gel electrophoresis or other methods. DNA may be extracted from the plants using conventional methods and the PCR reaction carried out using primers that will distinguish the transformed and non-transformed plants. For example, primers may be designed that will amplify a region of DNA from the transformation vector reading into the construct and the reverse primer designed from the gene of interest. These primers will only amplify a fragment if the plant has been successfully transformed. An alternative method to confirm a positive transformant is by Southern blot hybridization, well known in the art. Plants which are transformed or mutant may also be identified i.e. distinguished from non-transformed or wild-type plants by their phenotype, for example conferred by the presence of a selectable marker gene, or the presence of a particular protein by immunological methods, or by the absence of a protein, for example that absence of the SBEIIa protein in the endosperm as detected by ELISA assay. An indication used in screening such plants might also be by observation of the phenotypic traits of the grain, for example by visual inspection or measurement of shrunken grain, or testing for elevated amylose content, or checking microscopically for the presence of birefringence.

Mutation

Introduction of the genetic variatiion leading to reduced activity of the SBEIIa enzyme or other enzyme in the barley endosperm may also be achieved by the appropriate mutations within the respective gene or regulatory sequences of the gene. The extent to which the gene is inhibited will to some degree determine the characteristics of the starch made. The mutations may be truncation or null mutants and these are known to have a significant impact on the nature of the starch, however an altered amylopectin structure will also result from a leaky mutant that sufficiently reduces amylopectin synthesis enzyme activity to provide the characteristic of interest in the starch or grain of barley. Other chromosomal

rearrangements may also be effective and these might include deletions, inversions, duplication or point mutations.

Mutagenesis can be achieved by chemical or radiation means, for example EMS or sodium azide (Zwar and Chandler, 1995) treatment of seed, or gamma irradiation. Isolation of mutants may be achieved by screening mutagenised plants or seed. For example, a mutagenized population of barley may be screened for high amylose content in the grain and/or longer than normal amylopectin chain length distribution, or loss of the SBEIIa protein by ELISA, or for altered grain morphology (Green *et al.*, 1997). Screening is preferably done in a barley genotype which already lacks one of the SBE activities, for example in a SBEIIb-negative background. Such mutations may then be introduced into desirable genetic backgrounds by crossing the mutant with a plant of the desired genetic background and performing a suitable number of backcrosses to cross out the originally undesired parent background.

Mutations in the genes encoding the SBEIIa or other enzymes involved in amylopectin synthesis will generally cause increased relative amylose content. The amount of amylose per individual grain may be increased as a consequence of diverted carbon flow from amylopectin to amylose, or it may be decreased if there is a significant decrease in starch production per grain. In either case, the relative level of amylose as a percentage of starch increases.

Suitable for food production

In another aspect, the invention provides barley that is useful for food production, the grain being obtained from a barley plant having a reduced level of SBEIIa activity in the endosperm of developing grain, starch of said grain having a relatively high amylose content and a reduced amylopectin content. The barley plant of the present invention is preferably one having grain that is useful for food production and in particular for commercial food production. Such food production might include the making of flour or other products that might be an ingredient in commercial food production.

The desired genetic background of the barley will include considerations of agronomic yield and other characteristics. Such characteristics might include whether it is desired to have a winter or spring type of barley, agronomic performance, disease resistance and abiotic stress resistance. In Australia one might want to cross into barley cultivars such as Sloop,

Schooner, Chebec, Franklin, Arapiles, Tantangara, Galleon, Gairdner or Picola. The examples provided are specific for an Australian production region, and other varieties will be suited for other growing regions. It is preferred that the barley variety of the invention provide a yield not less than 80% of the corresponding wild-type variety in at least some growing regions, more preferably not less than 90% and even more preferably not less than 95%. The yield can readily be measured in controlled field trials. It is also preferred that the barley plants are hull-less or "naked", because the presence of husks on barley grains introduces greater difficulty in processing the grain.

The starch content of the grain should be at least about 12% (w/w) or 15%, preferably at least 25%, more preferably at least 35% and even more preferably near to the wild-type levels of 45-50% (w/w). Lower starch contents than wild-type are likely a consequence of reduced amylopectin levels. The grain may still be useful for commercial food production because of the relatively high value of the high amylose products. Other desirable characteristics include the capacity to mill the grain. Whilst pearled barley may be produced from most forms of grain, certain configurations of grain are particularly resistant to milling. Another characteristic that might have an impact on commercial usefulness of grain is the colouration of the product produced from the grain. Where the husk or other portion of the grain exhibits significant colouration other than the normal this may limit its commercial applications to niche applications such as being a component of bread containing coloured whole or kibbled grains. Typically in barley the significant colouration is purple, and that may be a bright and strong colouration which is highly undesirable in most food products. Another aspect that might make a barley plant of higher value is the degree of starch extraction from the grain, the higher extraction rates being more useful. Grain shape is also another feature the can impact on the commercial usefulness of a plant, thus grain shape can have an impact on the ease or otherwise with which the grain can be milled. For example, the barley grain of the high amylose MK6827 plant has a very elongated grain morphology which makes it difficult to mill and process. A convenient measure of this elongate shape and associated usefulness is the ratio of the length of the grain to the thickness of the grain (L/T ratio). This ratio is often dictated by the nature of the starch. It is preferred that this ratio is less than 5.5, more preferably ranging from about 4 to about 5, and most preferably less than 3.5 on average.

A fuller grain may be desirable in terms of achieving greater yields and certain benefits of the invention might be achieved, such as the production of starch with high levels of amylose, or in the alternative starch with altered chain length distributions. Thus the grain preferably has a non-shrunken phenotype. Other aspects of the invention may, however, be better achieved by a grain that is less filled. Thus the proportion of aleurone layer or germ to starch may be higher in less filled grain, thereby providing for a barley flour or other product that is higher in the beneficial constituents of the aleurone layer. The high aleurone layer product might thus be higher in certain vitamins such as folate, or it might be higher in certain minerals such as calcium, and that combined with higher resistant starch levels and/or higher β glucan levels might provide synergistic effects such as providing for enhanced uptake of minerals in the large bowel.

In order to maximise the amount of amylose, it may be desirable for the barley plant to also have other phenotypic characteristics in addition to a reduced activity of SBEIIa. The genetic background might therefore include additionally the *amo1* mutation in AC38 (causal gene unknown) or the waxy mutation (found for example in the Waxiro variety). Additionally it might be desired to make double mutations in other barley mutants available with shrunken endosperms where the causal gene is not known.

Starch is readily isolated from barley grain using standard methods, for example the method of Schulman *et al.* (1991). On an industrial scale, wet or dry milling can be used. The starch obtained from the grain of barley plant of the invention has a high relative amylose content. Barley plants having at least 35-45% amylose in the starch are considered to be high amylose. The present invention however provides for barley with an amylose content that is greater than 50% (w/w), preferably at least 60%, and more preferably at least 70%, 75%, 80% or 90%. .

It will be understood that the relative level of amylose referred to is in relation to total starch content, and thus the remainder of the starch might be predominantly of an intermediate type of starch or it might be predominantly amylopectin or a mixture of both.

β-Glucan

It is known that there is a wide variation in β glucan levels in barley in the range of about 4% to about 18% by weight of the barley, but more typically from 4% to about 8% (for example, Izydorcyk *et al.*, 2000). Enhanced barley strains have been developed, for example, which have between about 15% and about 18% by weight β-glucan but has a waxy phenotype.

The levels of β glucan contemplated by this invention may depend on the genetic background in which the amylopectin synthesis enzyme activity, including SBEIIa, is reduced. The exemplified embodiment shows relatively normal β glucan synthesis, however other forms of the invention may contemplate an elevated relative level of β glucan. Thus the grain of the barley plant preferably has a β glucan content of between about 3 to 6% (w/w) of total non-hulled grain weight. Other forms of the invention may however exhibit β-glucan content of greater than 6% or higher, for example, 6-8%. Levels of β glucan in a waxy mutant has been measured as being as high as 15 to 18%, for example variety Prowashonupana, sold commercially under the name Sustagrain™, (ConAgra™ Specially Grain Products Company, Omaha, Neb. USA) and the present invention may contemplate levels as high, or higher, than that.

Gelatinisation temperature

Gelatinisation is the collapse (disruption) of molecular order within the starch granule with concomitant and irreversible changes in properties such as granular swelling, crystallite melting, loss of birefringence, viscosity development and starch solubilisation. High amylose starch from *ae* (amylose extender) mutants of maize showed a higher gelatinisation temperature than normal maize (Fuwa et al., 1999, Krueger et al., 1987). On the other hand, starch from barley *sex6* mutants that lack starch synthase IIa activity had lower gelatinisation temperatures and the enthalpy for the gelatinisation peak was reduced when compared to that from control plants (Morell et al., 2003).

In another aspect of the invention, the starch may have an altered gelatinisation temperature as measured by differential scanning calorimetry. This may be either increased or reduced compared to starch from wild-type plants. The altered gelatinisation temperature may be in addition to the relatively high amylose content. Where the gelatinisation temperature is reduced, it may be reduced when compared to starch produced by other barley varieties with

elevated amylose content, or it may be reduced when compared with starch produced from barley with normal levels of amylose. Alternative forms of the invention contemplate gelatinisation temperatures that are unaltered or are raised relative to wild-type barley starch. The gelatinisation temperature of wild-type barley starch is typically about 56°C for the temperature of the first peak as measured by differential scanning calorimetry.

Swelling volume

The starch may also be characterized by its swelling rate in heated excess water compared to wild-type starch. Swelling volume is typically measured by mixing either a starch or flour with excess water and heating to elevated temperatures, typically greater than 90°C. The sample is then collected by centrifugation and the swelling volume is expressed as the mass of the sedimented material divided by the dry weight of the sample. A low swelling characteristic is useful where it is desired to increase the starch content of a food preparation, in particular a hydrated food preparation.

Crystallinity

The starch structure of the barley of selected forms of the present invention may also differ in that the degree of crystallinity is reduced compared to normal starch isolated from barley. The reduced crystallinity of a starch is also thought to be associated with enhance organoleptic properties and contributes to a smoother mouth feel. Thus the starch may additionally exhibit reduced crystallinity resulting from reduced levels of activity of one or more amylopectin synthesis enzymes. Crystallinity is typically investigated by X-ray crystallography.

Distribution of amylopectin chain lengths

One measurement of an altered amylopectin structure is the distribution of chain lengths, or the degree of polymerization, of the starch. The chain length distribution may be determined by using fluorophore-assisted carbohydrate electrophoresis (FACE) following isoamylase de-branching. The amylopectin of the starch of the invention may have a distribution of chain length in the range from 5 to 60 that is greater than the distribution of starch from wild-type plants upon debranching. Starch with longer chain lengths will also have a commensurate decrease in frequency of branching. Thus the starch may also have a distribution of longer amylopectin chain lengths in the amylopectin still present.

Food characteristics

Starch is the major source of carbohydrate in the human diet, and the grain of the invention and products derived from it can be used to prepare food. The food may be consumed by man or animals, for example in livestock production or in pet-food. The grain derived from the altered barley plant can readily be used in food processing procedures, and therefore the invention includes milled, ground, kibbled, pearled or rolled grain or products obtained from the processed or whole grain of the barley plant referred to above, including flour. These products may be then used in various food products, for example farinaceous products such as breads, cakes, biscuits and the like, or food additives such as thickeners or binding agents, or to make malted or other barley drinks, noodles and quick soups. The grain or products derived from the grain of the invention are particularly desired in breakfast cereals. The high amylose starches of the invention can also be used to form high strength gels which are useful in the confectionery industry, or allow lower molding and curing times. They may also be used as a coating, for example to reduce oil absorption in deep-fried potato or other foods.

Dietary fibre

Dietary fibre, in this specification, is the carbohydrate and carbohydrate digestion products that are not absorbed in the small intestine of healthy humans but enter the large bowel. This includes resistant starch, β-glucan and other soluble and insoluble carbohydrate polymers. It is intended to comprise that portion of carbohydrates that are fermentable, at least partially, in the large bowel by the resident microflora.

The starch of the invention preferably contains relatively high levels of dietary fibre, more particularly amylose and optionally an elevated level of β-glucan. The dietary fibre content of the grain of the present invention may or may not result solely from the increased relative endospermal amylose content. The β-glucan may be present at elevated levels and as such may contribute significantly to the dietary fibre level.

Aspects of this invention might also arise from the combination of aleurone layer and germ in combination with high levels of dietary fibre. Specifically, this may arise where higher relative levels of aleurone or germ are present in the grain. Firstly, barley has a significantly higher aleurone layer than other commercial grains, being a result of having a three cell aleurone layer. Secondly, where the barley grain is slightly shrunken the endosperm is present in reduced amounts and the aleurone layer and the germ are present in relatively

elevated amounts. Thus the barley has a relatively high level of certain beneficial elements or vitamins in combination with elevated resistant, such elements include divalent cations such as bioavailable Ca^{++} and vitamins such as folate or antioxidants such as tocopherols and tocotrienols. Calcium is required for growth and deposition of bone and other calcified tissue and in lowering the risk of osteoporosis later in life. Folic acid is found to be protective against neural tube defects when consumed periconceptually and decreases the risk of cardiovascular disease, thereby enhancing the effects of the combination of resistant starch and β-glucan. Folic acid also is thought to have an effect of lowering the risk of certain cancers. Tocopherol and tocotrienols carry the benefits of antioxidants and are believed to lower the risk of cancer and heart disease, and also have the effect of reducing the undesirable effects of oxidation of components of a food such as fatty acids which can result in rancidity. One specific form of milled product might be one where the aleurone layer is included in the milled product. Particular milling process might be undertaken to enhance the amount of aleurone layer in the milled product. Such a method is referred to in Fenech *et al.* (1999). Thus any product derived from grain milled or otherwise processed to include aleurone layer and germ will have the additional nutritional benefits, without the requirement of adding these elements from separate sources.

Resistant starch

Resistant starch is defined as the sum of starch and products of starch digestion not absorbed in the small intestine of healthy humans but entering into the large bowel. Thus, resistant starch excludes products digested and absorbed in the small intestine. Resistant starches include physically inaccessible starch (RS1 form), resistant granules (RS2), retrograded starches (RS3), and chemically modified starches (RS4).

The altered starch structure and in particular the high amylose levels of the starch of the invention give rise to an increase in resistant starch when consumed in food. Resistant starch may also increase if β-glucan is present at elevated levels, which is likely to exert protective effects by association of the β glucan with the starch granule. The starch may be in an RS1 form, being somewhat inaccessible to digestion. Starch-lipid association as measured by V-complex crystallinity is also likely to contribute to the level of resistant starch. In this case the resistance is likely to arise because of the physically inaccessible of the starch by virtue of the presence of the lipid and accordingly this might be regarded as an

RS1 starch. The starch of the exemplified barley plant may be resistant to digestion by reason of the structure of the starch granule and accordingly may have RS2 starch. Each of these characteristics might be present separately or in combination.

It will be understood that one benefit of the present invention is that it provides for products that are of particular nutritional benefit, and moreover it does so without the need to modify the starch or other constituents of the barley grain. However it may be desired to make modifications to the starch, β–glucan or other constituent of the grain, and the invention encompasses such a modified constituent. Methods of modification are well known and include the extraction of the starch or β–glucan or other constituent by conventional methods and modification of the starches to increase the resistant form. The starch or β-glucan may be modified by treatment with heat and/or moisture, physically (for example ball milling), enzymatically (using for example α– or β-amylase, pullalanase or the like), chemical hydrolysis (wet or dry using liquid or gaseous reagents), oxidation, cross bonding with difunctional reagents (for example sodium trimetaphosphate, phosphorous oxychloride), or carboxymethylation.

Glycemic index

Glycaemic Index (GI) is a comparison of the effect of a test food with the effect of white bread or glucose on excursions in blood glucose concentration. The Glycaemic Index is a measure of the likely effect of the food concerned on post prandial serum glucose concentration and demand for insulin for blood glucose homeostasis. One important product provided by the invention as a result of the high amylose and optionally high β-glucan content is a low calorific product with a reduced glycaemic index. A low calorific product might be based on inclusion of flour produced from milled barley grain. It might be desired, however, to first pearl the grain removing perhaps 10% or 20% by weight of the grain, thereby removing the aleurone layer and at the greater reduction removing also the germ. The effect of the pearling step is to reduce the lipid content and therefore reducing the calorific value of the food. Such foods will have the effect of being filling, enhancing bowel health, reducing the post-prandial serum glucose and lipid concentration as well as providing for a low calorific food product. Use of the pearled product would result in a reduction in nutritional benefits provided by the aleurone layer and the germ. The flour

produced from the pearled product is likely to have an enhanced appearance because a product made in that way tends to be whiter.

Non-food applications

The present invention provides modified or improved starches having elevated levels of amylose or reduced levels of amylopectin whose properties satisfy any of various industrial requirements. Starch is widely used in non-food industries, including the paper, textile, corrugating and adhesive industries (Young, 1984). The physical properties of unmodified starch limits its usefulness in some applications and often imposes a requirement for chemical modification that can be expensive or have other disadvantages. The invention provides starch for which less post-harvest modification may be required, in particular due to the reduced amylopectin content in combination with other physical properties. For example, the pasting temperature, resistance to shearing stresses, film strength and/or water resistance of starches and product made from the grain of this invention may be altered. The starch may also be used to prepare a biodegradable loose-fill packing material that can be used as a replacement for polystyrene.

It will be understood that whilst various indications have been given as to aspects of the present invention, the invention may reside in combinations of two or more aspects of the present invention.

EXAMPLES

EXAMPLE 1. MATERIALS AND METHODS

Callus inducing medium

BCI-DM medium containing Dicamba (2.5 mg/l) was used for callus induction from barley embryo. Composition for one litre of medium:

MS salt Macro (10x stock):	100 ml
MS micro (100x stock):	10 ml
Iron (200x stock):	5 ml
EDTA (200x stock):	5 ml
Maltose:	15.0 g
Thiamine-HCl (1mg/ml):	1 ml
Myo-inositol:	250 mg

Casein hydrolysate:	1 g
Dicamba (1mg/ml):	2.5 ml
Proline:	345 mg

The pH was adjusted to 5.8 and 3.5 g/l of Phytagel added. After autoclaving the medium, 150 mg/l of Timentin and 50mg/l of Hygromycin were added.

Barley regeneration medium

Barley calli are regenerated in FHG medium containing BAP (1 mg/l)

FHG-I Macro (10x stock):	100 ml
FHG-II Micro (100x stock):	10 ml
Thiamine-HCl (1mg/ml):	1 ml
Iron (200x stock):	5 ml
EDTA (200x stock):	5 ml
BAP (1 mg/ml):	1 ml
Inositol:	100 mg
Glutamine:	730 mg
Maltose:	62 g

The pH was adjusted to 5.8 and then 3.5 g/l of phytagel added. After autoclaving the medium,150 mg/l of Timentin and 20 mg/l of hygromycin were added.

Carbohydrate determination and analysis

Starch was isolated from barley grain using the method of Schulman *et al.* (1991). Starch content was determined using the total starch analysis kit supplied by Megazyme (Bray, Co Wicklow, Republic of Ireland). The starch content is then compared to control plants. Subtraction of the starch weight from the total grain weight to give a total non-starch content of the grain determines whether the reduction in total weight is due to a reduction in starch content.

Determination of the amylose content or the amylose/amylopectin ratio was performed by an HPLC method for separating debranched starches or by an iodine binding method, as described by Batey and Curtin (1996). Briefly, starch was defatted by dissolving it in DMSO and reprecipitation with ethanol. After redissolving the starch in DMSO and the

addition of water, further dilution, and addition of an iodine/potassium iodide solution, the absorbance of the solution was measured at 605nm. The amylose content was determined from a standard curve obtained from mixtures of amylose and amylopectin covering the range 0-100% amylose. Analysis of the amylose/amylopectin ratio of non-debranched starches may also be carried out according to Case *et al.*, (1998).

β-Glucan levels were determined using the kit supplied by Megazyme (Bray, Co Wicklow, Republic of Ireland).

Starches were debranched and chain length distributions analysed using fluorophore assisted carbohydrate electrophoresis (FACE) using a capillary electrophoresis unit according to Morell *et al* (1998).

Differential Scanning Calorimetry (DSC)

DSC measures the changes to gelatinisation temperatures that have occurred in the starch by changes in amylose and amylopectin ratio. Gelatinisation was measured in a Pyris 1 differential scanning calorimeter (Perkin Elmer, Norwalk CT, USA). Starch was mixed with water in the ratio of 2 parts water: 1 part starch and this mixture (40-50 mg, accurately weighed) placed in a stainless steel pan and sealed. The sample was scanned at 10°C per minute from 20°C to 140°C with an empty stainless steel pan as a reference. Gelatinisation temperatures and enthalpy were determined using the Pyris software.

RVA Analysis

Viscosity was measured on a Rapid-Visco-Analyser (RVA, Newport Scientific Pty Ltd, Warriewood, Sydney) using conditions as a reported by Batey et al., 1997 for wholemeal flours. In order to inhibit α-amylases, silver nitrate was included in all assays at a concentration of 12 mM. The parameters measured were peak viscosity (the maximum hot paste viscosity), holding strength, final viscosity and pasting temperature.

Flour Swelling

Flour swelling volume was determined according to the method of Konik-Rose *et al* (2001). Increased uptake of water was measured by weighing the sample prior to and after mixing the sample in water at defined temperatures and following collection of the gelatinized material.

EXAMPLE 2 ISOLATION OF SBE GENES FROM BARLEY

Construction of barley cDNA and genomic libraries.

Barley cDNA and genomic libraries were made by standard methods in phage vectors (Sambrook et al, 1989). A cDNA library was made in the ZipLox vector (Life Technology) according to the protocols supplied with the reagents. The titre of the library was 2×10^6 pfu tested with Y1090(ZL) strain of *E. coli.* The barley genomic library, obtained from E. Lagudah (CSIRO), was made from DNA from the variety Morex. The DNA was digested with *Mbo*I and ligated to *Eco*RI/*Bam*HI digested EMBL3cos vector. Cloned fragments could be released with *Sal*I digestion.

Isolation of SBEIIa and SBEIIb gene sequences from a H. vulgare genomic library

Conditions for library screening were hybridisation at 25% formamide, 5 x SSC, 0.1% SDS, 10x Denhardts solution, 100μg/ml salmon sperm DNA at 42°C for 16hr, followed by washing with 2 x SSC, 0.1% SDS at 65°C for 3 x 1hr (medium stringency). Clones containing the *SBEIIa* and *SBEIIb* genes or substantial portions thereof were isolated and sequenced. DNA sequence comparisons to those of the Accession Nos. listed in Table 1 confirmed that both genes of interest had been isolated from barley. SBEIIa and SBEIIb cDNA sequences may also be obtained using reverse transcription-PCR (RT-PCR) with specific primers, a technique well known in the art. Barley SBEIIa and SBEIIb cDNA sequences are shown in Figures 1 and 2, and wheat SBEIIa and SBEIIb genomic sequences shown in Figures 3 and 4.

Table 1. Starch branching enzyme genes characterized from cereals

Species	SBE isoform	Type of clone	Accession No.	Reference
Maize	SBE I	cDNA	U17897	Fisher et al., 1995
		genomic	AF072724	Kim et al., 1998a
	SBE IIb	cDNA	L08065	Fisher et al., 1993
		genomic	AF072725	Kim et al., 1998
	SBE IIa	cDNA	U65948	Gao et al., 1997
Wheat	SBE II	cDNA	Y11282	Nair et al., 1997
	SBE I	cDNA and genomic	AJ237897 SBE I gene) AF002821 (SBE I pseudogene AF076680 (SBE I gene) AF076679 (SBE I cDNA)	Baga et al., 1999 Rahman et al., 1997, Rahman et al., 1999
	SBE I	cDNA	Y12320	Repellin et al., 1997
	SBE IIa	cDNA and genomic	AF338432 (cDNA) AF338431 (gene)	Rahman et al., 2001
Rice	SBE I	cDNA	D10752	Nakamura and Yamanouchi, 1992
	SBE I	genomic	D10838	Kawasaki et al., 1993
	SBE3	cDNA	D16201	Mizuno et al., 1993
Barley	SBE IIa and SBE IIb	cDNA and genomic	AF064563 (SBE IIb gene) AF064561 (SBE IIb cDNA) AF064562 (SBE IIa gene) AF064560 (SBE IIa cDNA)	Sun et al., 1998

EXAMPLE 3: CONSTRUCTS FOR TRANSFORMATION EXPERIMENTS TO ALTER BARLEY SBEIIA AND SBEIIB EXPRESSION.

Duplex-RNA (dsRNA) constructs were made to reduce the expression of either the *SBEIIa* or *SBEIIb* genes of barley. In such constructs, the desired nucleic acid sequence corresponding to part of the *SBEIIa* or *SBEIIb* genes occurred in both the sense and antisense orientations relative to the promoter so that the expressed RNA comprised complementary regions that were able to basepair and form a duplex or double-stranded RNA. A spacer region between the sense and antisense sequences comprised an intron sequence which, when transcribed as part of the RNA in the transformed plant, would be spliced out to form a tight "hairpin" duplex structure. The inclusion of an intron has been found to increase the efficiency of gene silencing conferred by duplex-RNA constructs (Smith et al, 2000). The desired nucleic acid was linked to a high molecular weight glutenin (HMWG) promoter sequence (promoter of the DX5 subunit gene, Accession No. X12928, Anderson et al., 1989) and terminator sequence from the nopaline synthase gene from *Agrobacterium* (nos3').

Duplex-RNA constructs containing SBEIIa or SBEIIb sense/antisense fragments, obtained from wheat *SBEIIa* and *SBEIIb* genes in view of the high degree of sequence identity between the wheat and barley genes, were initially generated in the vector pDV03000 and then cut out and ligated to the barley transformation vector pWBVec8. The constructs are shown schematically in Figure 5. The vector pWBVec8 contains a number of restriction enzyme sites for incorporation of desired DNA sequences.

The SBEIIa duplex-RNA construct contained 1536bp of nucleotide sequence amplified by PCR from the wheat SBEIIa gene (GenBank Accession number AF338431, see Figure 3). This included; a 468bp sequence that comprises the whole of exons 1 and 2 and part of exon 3 (nucleotide positions 1058 to 1336, 1664 to 1761 and 2038 to 2219 in Figure 3), with *Eco*RI and *Kpn*I restriction sites on either side (fragment 1), a 512bp sequence consisting of part of exons 3 and 4 and the whole of intron 3 of *SBEIIa* (nucleotide positions 2220 to 2731 in Figure 3) with *Kpn*I and *Sac*I sites on either side (fragment 2) and a 528bp fragment consisting of the complete exons 1, 2 and 3 of *SBEIIa* (nucleotide positions 1058 to 1336, 1664 to 1761 and 2038 to 2279 in Figure 3) with *Bam*HI and *Sac*I sites on either side (fragment 3). Fragments 1, 2 and 3 were then ligated so that the sequence of fragment 3 was ligated to fragment 2 in the antisense orientation relative to

fragment 1. The gene construct in the vector pDV03000 was designated pDV03-IIa and the duplex-RNA gene designated ds-SBEIIa.

The strategy for the SBEIIb duplex-RNA construct was similar. The SBEIIb construct contained a fragment of 1607bp amplified by PCR from the wheat *SBEIIb* gene (sequence is outlined in Figure 4). This included; a 471bp sequence that comprises the whole of exons 1 and 2 and part of exon 3 (nucleotide positions 489 to 640, 789 to 934 and 1598 to 1769 in Figure 4), with *Eco*RI and *Kpn*I restriction sites on either side (fragment 1), a 589bp sequence consisting of part of exons 3 and 4 and the whole of intron 3 of *SBEIIb* (nucleotide positions 1770 to 2364 in Figure 4) with *Kpn*I and *Sac*I sites on either side (fragment 2) and a 528bp fragment consisting of the complete exons 1, 2 and 3 of *SBEIIb* (nucleotide positions 489 to 640, 789 to 934 and 1598 to 1827 in Figure 4) with *Bam*HI and *Sac*I sites on either side (fragment 3). Fragments 1, 2 and 3 were then ligated so that the sequence of fragment 3 was ligated to fragment 2 in the antisense orientation relative to fragment 1. The SBEIIb duplex-RNA gene construct in the vector pDV03000 was designated pDV03-IIb and the duplex-RNA gene designated ds-SBEIIb.

The promoter-sense/antisense-terminator cassettes were inserted into the binary vector pWBVec8 using the restriction enzymes *Apa*I and *Not*I. The SBEIIa construct in pWBVec8 was designated pVec8-IIa and the SBEIIb construct in pWBVec8 was designated pVec8-IIb. The constructs are shown schematically in Figure 5.

The identity between the wheat SBEIIa sequences used and the corresponding barley SBEIIa sequence was 93% using the program Gap to compare the sequences. Similarly, the identity between the wheat SBEIIb sequence and the corresponding barley SBEIIb sequence was 92%. Duplex-RNA technology is effective for silencing the expression of genes having sequences with identities over about 85% with respect to the duplex region, and so the expectation was that the duplex constructed with the wheat sequences would be effective against the barley sequences.

EXAMPLE 4: TRANSFORMATION OF BARLEY.

Methods for the transformation of barley, mediated by *Agrobacterium tumefaciens* or by biolistics, have been described (Tingay *et al.*, 1997; Wan *et al,* 1994) and can be used to transfer DNA constructs generating transgenic plants. In this example, the gene constructs

in binary vectors, made as described above, were introduced into a highly virulent *Agrobacterium* strain by tri-parental conjugation, which was then used to introduce the T-DNA containing the inhibitory gene (ds-SBEIIa or ds-SBEIIb) and the selectable marker gene (encoding hygromycin resistance, expressed from the CaMV35S promoter) into regenerable cells of the scutellum of immature barley embryos, as follows.

Developing barley seeds from the variety Golden Promise, 12-15 days after anthesis, were removed from the growing spike of greenhouse grown plants, and sterilised for ten minutes in 20% (v/v) bleach followed by rinsing once with 95% ethanol and seven times with sterile water. Embryos (approx 1.5 to 2.5mm in size) were then removed from the seeds under aseptic conditions and the axis cut from each embryo. The embryos were placed cut side down on a petri dish containing callus induction medium. The *Agrobacterium* transconjugants (strain AGL1) were grown in MG/L broth (containing 5 g mannitol, 1 g L-glutamic acid, 0.2 g KH_2PO_4, 0.1 g NaCl, 0.1 g $MgSO_4.7H_2O$, 5 g tryptone, 2.5 g yeast extract and 1 μg biotin per litre, pH 7.0) containing spectinomycin (50 mg/l) and rifampicin (20 mg/l) with aeration at 28°C, to a concentration of approximately 2-3 x 10^8 cells/ml, and then approx 300 μl of the cell suspension was added to the embryos in a petri dish. After 2 min, excess liquid was tipped from the plate and the embryos were flipped so that the cut side (axil side of the scutellum) was upwards. The embryos were then transferred to a fresh plate of callus inducing medium and placed in the dark for 2-3 days at 24°C. The embryos were transferred to callus inducing medium with selection (50 μg/ml hygromycin and 150 μg/ml timentin). Embryos remain on this media for 2 weeks in the dark at 24°C. Healthy callus was then divided and placed on fresh selection media and incubated for a further two weeks at 24°C in the dark. Following this, the embryos were incubated at 24°C in the light for 2 weeks on regeneration medium containing cytokinin and transferred to rooting media containing cytokinin and auxin for three 2 week periods. Juvenile plants were then transferred to soil mixture and kept on a misting bench for two weeks and finally transferred to a glasshouse. A total of 400 embryos using pVec8-IIb and 300 embryos using pVec8-IIa were treated by this method and 18 plants from 7 calli for the IIb transformation and 18 plants from 14 calli for the IIa transformation survived on selection medium, suggesting that they were successfully transformed with the gene construct. Not all of the plants that were transformed with the selectable marker gene would be expected to integrate the SBEIIa or SBEIIb inhibitory gene; these could readily be distinguished as described in the following examples.

EXAMPLE 5. ANALYSIS OF BARLEY PLANTS AND GRAIN TRANSFORMED WITH DUPLEX-RNA CONSTRUCTS.

The presence or absence of the transgene(s) in barley plants or progeny seed or plants was determined or confirmed by PCR techniques or Southern blot hybridisation analysis. DNA was prepared from leaf samples from putative transformed plants by standard methods.

PCR analysis of transformed barley plants – detection of transgenes.

The forward and reverse primers used for screening the presence of the ds-SBEIIa transgene were BX17 3' (5'- CAA CCA TGT CCT GAA CCT TCA CC-3') SEQ ID No. 5 and AR2akpnR (5'-GGT ACC CCA TCT CCT GGT TTT GGG ACA AC-3') SEQ ID No. 6, respectively. This primer pair amplified a 569bp product, corresponding to a position within the HMWG promoter sequence of the transgene to the nucleotide position 2219 in Figure 3, from those plants containing the ds-SBEIIa transgene. The primers used for screening for the presence of the ds-SBEIIb transgene were BX17 3' (as above) and AR2bkpnR (5'-GGT ACC GTC CAT TTC CCG GTG GTG GCA G-3') SEQ ID No. 7. This primer pair amplified a 571bp product, corresponding to a position within the HMWG promoter to nucleotide position 1768 in Figure 4, from those lines containing the ds-SBEIIb transgene. PCR amplification was conducted in a 20 µl reaction containing 2.5 units Hotstar Taq, 1 x buffer supplied with the enzyme containing 1.5 mM $MgCl_2$, 0.125 mM each deoxynucleotide triphosphate (dNTPs), 1 µM each of the forward and reverse primers and 100 ng DNA. The PCR programme included an initial denaturation step of 95°C for 5 min, followed by 36 cycles of 95°C for 30sec, 59°C for 1 min and 72°C for 2 min, finished with 72°C for 5 min.

Positive barley transformants were identified for both of the SBEIIa and SBEIIb constructs (Figure 6). The data is summarized in Table 2.

Table 2. Summary of PCR and Southern hybridization results of SBEIIa and SBEIIb transgenic lines of barley.

SBEIIb transgenic line No.	Transform. event no.[a]	PCR	Southern	SBEIIa transgenic line No.	Transform. event no.[a]	PCR	Southern
IIb1.1	1	-	-	IIa1.1	1	-	-
IIb1.2	1	-	-	IIa2.1	2	-	-
IIb1.3	1	+	+ (vf)	IIa3.1	3	+	-
IIb2.1	2	+	+	IIa3.2	3	+	-
IIb2.2	2	+	+	IIa4.1	4.1	+	+
IIb3.1	3	+	+	IIa4.2	4.2	+	+
IIb4.1	4	+	+	IIa5.1	5	+	nr
IIb4.2	4	+	+	IIa5.2	5	+	+
IIb4.3	4	+	+	IIa6.1	6	+	+
IIb4.4	4	+	+	IIa6.2	6	+	+
IIb4.5	4	+	+	IIa7.1	7	-	-
IIb4.6	4	-	+	IIa9.1	9	+	nr
IIb5.1	5	+	+ (f)	IIa10.1	10	+	nr
IIb8.1	8	+	-	IIa11.1	11	-	-
IIb8.3	8	+	-	IIa13.2	13	+	nr
IIb8.4	8	+	+ (f)	IIa13.3	13	+	nr
IIb9.1	9	+	+	IIa15.1	15	+	nr
				IIa16.1	16	-	-

a: Transformation event Nos. with the same number were isolated from the same callus and may be identical or independent. Different numbers: independent transformants.

(f): faint; (vf): very faint; nr: no result

Southern blot hybridization analysis of transformed barley.

Southern blot hybridization analysis was carried out on DNA from the ds-SBEIIa and ds-SBEIIb transgenic plants and their progeny to confirm the PCR results. *Eco*R1 digested DNA, prepared from the plants by standard methods, was electrophoresed on 1% agarose gels and blotted on to Hybond N+ nylon membrane (Amersham). Radio-labelled probes were generated from the intron 3 region of the *SBEIIa* (positions 2220 to 2731 see Figure 3) and *SBEIIb* (positions 2019 to 2391 see Figure 4) genes. These segments are part of the respective ds-SBEIIa and ds-SBEIIb constructs (Example 3) and were radioactively labeled using the Megaprime DNA labeling system (Amersham Pharmacia Biotech UK Ltd) and

used for hybridization. The hybridization was carried out in 25% (v/v) formamide, 5 x SSC, 0.1% SDS, 10 x Denhardt's solution, 100 μg/ml salmon sperm DNA at 42°C for 16 hr followed by washing in 2 x SSC, 0.1% SDS at 65°C for 3 x 1hr. Autoradiography of the membranes revealed positive hybridizing bands in lanes corresponding to plants that were positive for the constructs (Figure 7). The endogenous barley SBEIIa and SBEIIb gene fragments were not detected in the hybridization because of sequence divergence with the wheat intron 3 probe used.

The results of the PCR and Southern hybridization analyses are summarized in Table 2. In general, the PCR and Southern hybridization results correlated well. Discrepancies may have been due to false negatives and would readily be resolved by repeated assays. Plants that were positive for the transgenes as demonstrated by both methods included 4 independent transformation events for ds-SBEIIa Southern (IIa 4.1, IIa 4.2, IIa 5 and IIa 6) and 5 independent events for ds-SBEIIb (Event no. IIb 2, IIb3, IIb4, IIb5 and IIb 9).

Analysis of barley endosperm proteins by polyacrylamide gel electrophoresis (PAGE). To determine the effect of the ds-SBEIIa and ds-SBEIIb transgenes on the barley *SBEIIa* and *SBEIIb* gene expression in the transformed plants, specific protein expression in endosperm tissue of developing grains was detected by non-denaturing PAGE and Western blot analysis. Since the T1 seeds (seeds from T0 plants) were expected to be segregating for the transgenes, endosperm from each of ten individual developing T1 grains from each T0 plant, at 20 days after flowering, were analyzed for SBEIIa and SBEIIb protein expression. To preserve the T1 plants, embryos were rescued from the developing grains and cultured to regenerate the T1 plants. Endosperm dissected away from all maternal tissues (0.2 g) was homogenized in 600 μl of 50 mM KPi buffer (42 mM K_2HPO_4 and 8 mM KH_2PO_4), pH 7.5 containing 5 mM EDTA, 20% glycerol, 5 mM DTT and 1 mM Pefabloc. The ground samples were centrifuged for 10 min at 13,000g and the supernatant aliquoted and frozen at –80°C until use. Protein levels were measured with Coomassie reagent with BSA as a standard. Total soluble proteins, equivalent to 20 μg, extracted from each endosperm, were loaded per lane and electrophoresed in 8% non-denaturing polyacrylamide gels containing 0.34 M Tris-HCl (pH 8.8), acrylamide (8.0%), ammonium persulphate (0.06%) and TEMED (0.1%). Following electrophoresis, the proteins were transferred to a nitrocellulose membrane according to Morell et al., (1997) and immunoreacted with SBEIIa or SBEIIb specific antibodies. The antibody used for detection

of SBEIIa was 3KLH, from rabbits, which had been raised against the synthetic peptide AASPGKVLVPDESDDLGC SEQ ID No. 8 (the sequence from the N-terminus of SBEIIa), and was diluted 1:5000 for use. The antibody used for detection of SBEIIb was R6, raised against the synthetic peptide AGGPSGEVMIGC SEQ ID No. 9 (the deduced sequence from the N-terminus of SBEIIb) and diluted 1:6000 before use. The secondary antibody used was GAR-HRP conjugate (1:3000 dilution), and immunoreactive bands were revealed using an Amersham ECL-detection system.

The protein expression in the developing T1 seeds from plants transformed with the ds-SBEIIa or ds-SBEIIb genes appeared to be segregating in a 1:2:1 ratio of strong bands: moderate-weak bands: no bands for some of the transformed lines (for example, see Figures 8 and 9). This ratio corresponds to the expected segregation ratio of homozygotes (wild type = null for transgene): heterozygotes: homozygous for the transgene. The T1 plants from the rescued embryos are grown to produce T2 seed which are screened by PCR and protein expression analysis to confirm the genetic status of the T1 seed with respect to the transgene.

These data indicate that the duplex-RNA constructs are effective in reducing the expression of the *SBEIIa* and *SBEIIb* genes in endosperm of barley.

The expression of the *SBEIIb* gene in transgenic seeds containing the ds-SBEIIa transgene, and the expression of the *SBEIIa* gene in seeds containing the ds-SBEIIb were also analyzed by the Western blot method. Unexpectedly, transgenic seeds comprising ds-SBEIIa, for example from the transformation event IIa 4.1, were much reduced for SBEIIb. See Figure 9 which shows only a low level of expression of SBEIIb in seeds from line IIa 4.1.8 (note the very weak bands in 4 of the 7 lanes). This line contained the ds-SBEIIa transgene and had negligible SBEIIa expression. However, the converse effect was not observed in seeds transgenic for ds-SBEIIb. The SBEIIa expression was unaltered in the seeds in which SBEIIb was completely silenced by ds-SBEIIb (Figure 10) namely, for transgenic lines from the transformation events IIb 4 and IIb 2. The region including exons 1-3 was used for both ds-SBEIIa and ds-SBEIIb duplex constructs. Alignment of the sequences of *SBEIIa* and *SBEIIb* in this region revealed only ~70% identity. The longest stretch of 100% identity was a region of 21bp in the exon 2. Although it is still possible that expression of *SBEIIb* was suppressed by the ds-SBEIIa construct due to sequence

homology, it is also possible that the activity of SBEIIb was reduced by the ds-SBEIIa transgene by some other mechanism.

The expression levels of the *SBEIIa* and *SBEIIb* genes can also be specifically determined at the mRNA levels through standard techniques such as Northern hybridisation or RT-PCR methods, for example by using probes from non conserved regions or primer pairs which hybridize to unique sites in one of the genes but not the other, for example in the 3' untranslated regions. Such regions or sites can readily be identified by comparison of the two gene sequences.

EXAMPLE 6. ANALYSIS OF GRAIN COMPOSITION AND CONTENT, INCLUDING STARCH.

The grain composition and content, particularly for starch, may be measured using standard techniques such as those described in Example 1.

After extraction of the soluble proteins as described above, the starch granules from individual endosperm samples from developing seeds containing the ds-SBEIIa transgene were visualized under a light microscope. A significant alteration in starch granule morphology was observed (see for Example Figure 11) in developing endosperm that were reduced in SBEIIa expression for three of the five transformation events examined: IIa 4.1, IIa 4.2 and IIa 13, but not for events IIa 5 or IIa 6 which may have had a lesser degree of gene inactivation. For example, starch from IIa 4.2.5 seeds, which had no SBEIIa band in the protein immunoblot, was highly distorted compared to the normal granules in IIa 4.2.3 seeds, which had a strong SBEIIa band in the protein immunoblot (Table 3). Light microscopy results were confirmed by scanning electron microscopy (SEM), which may also be used to view starch granules directly. To do this, purified starch was sputtered with gold and scanned at 15 kV at room temperature. Seeds reduced for SBEIIa expression showed a distorted irregular shape that was visible under the scanning electron microscope, for example the distortion of granules in IIa 4.2.5 seeds compared to IIa 4.2.3 seeds (Figure 12).

In contrast to the plants containing ds-SBEIIa, the plants transformed with ds-SBEIIb showed endosperm starch granules with normal morphology when examined by microscopy, for example line IIb 4.1 (see Table 3). This suggests that reduction of *SBEIIb* expression alone did not substantially alter starch granule morphology.

Table 3. Starch granule morphology of T1 endosperm tissues of barley ds-SBEIIa and ds-SBEIIb transgenic lines

No	Transgenic line	Protein band on immunoblot	Starch granule morphology (light microscopy)
1	IIa 4.1.8	No band	Distorted
2	IIa 4.1.4	Strong band	normal
3	IIa 4.1.3	Strong band	normal
4	IIa 4.2.1	No band	Distorted
5	IIa 4.2.9	No band	distorted
6	IIa 4.2.5	No band	distorted
7	IIa 6.2.8	No band	normal
8	IIa 5.2.3	No band	normal
9	IIa 6.2.2	Strong band	normal
10	IIa 4.2.3	Strong band	normal
11	IIa 13.1.9	No band	normal
12	IIa 13.1.10	Weak band	normal
13	IIa 13.1.3	Strong band	normal
14	IIa 13.2.4	No band	Some distortion
15	IIa 13.1.6	Weak band	normal
16	IIb 4.1.9	No band	normal
17	IIb 4.1.8	No band	normal
18	IIb 4.1.2	No band	normal

Birefringence is the ability of a substance to refract light in two directions; this produces a dark cross called a "maltese cross" on each starch granule when viewed with a polarizing microscope. Birefringence is an indicator of the degree of ordered structural organization of the polymers within the granules (Thomas and Atwell, 1999). Starch granules from endosperm of IIa 4.2.5 seeds (reduced for SBEIIa activity) under polarized light indicated that there is a significant loss of birefringence in these granules compared to that from IIa 4.2.3 seeds (wild type) On average, 44.8% of the granules in IIa 4.2.5 seeds were without birefringence in contrast to 2.2% in IIa 4.2.3 seeds (Table 4). Loss of birefringence in starch granules is generally well correlated with increased amylose content.

Table 4. Birefringence of starch granules from T1 endosperm of ds-SBEIIa barley transgenic lines

Line	Microscopic field	No. of granules showing no BF	No. of granules showing partial BF	No. of granules showing full BF
A4.2.5 (SBEIIa negative)	1	38	19	12
	2	48	22	9
	2	26	25	35
	4	17	12	25
Total		129 (44.8%)	78 (27.1%)	81 28.1%)
A4.2.3 (control)	1	5	8	205
	2	3	9	104
	3	3	5	200
	4	2	2	85
Total		13 (2.1%)	24 (3.8%)	593 (94.1%)

BF: Birefringence

Grain weight analysis of transgenic seeds, from plants grown in the greenhouse, from the line IIa 4.2 containing ds-SBEIIa, revealed that there was no significant reduction in grain weight and therefore starch production, even in the seeds with highly distorted starch granules (Table 5). This is in contrast to the reduced grain weight observed in barley that is mutant in the *SSIIa* gene, which shows significantly reduced starch production (Morell et al, 2003). This suggests that the average grain weight and therefore the yield of field-grown barley with reduced SBEIIa activity in the endosperm is about normal.

Table 5. Grain weight of T1seeds from the SBE IIa barley transgenic line IIa 4.2

No.	Seed from line No.:	Starch granule morphology	Grain weight (mg)
1	IIa 4.2.1	Normal	46.4
2	IIa 4.2.2	Highly distorted	39.3
3	IIa 4.2.3	Distorted	39.0
4	IIa 4.2.4	Distorted	40.8
5	IIa 4.2.5	Highly distorted	37.3
6	IIa 4.2.6	Normal	41.8
7	IIa 4.2.7	Normal	35.0
8	IIa 4.2.8	Highly distorted	41.5
9	IIa 4.2.9	Highly distorted	41.1
10	IIa 4.2.10	Highly distorted	38.6

Amylose and amylopectin levels in transgenic barley grain.

Seed with starch granules having a distorted shape have been reported in high amylose barley (Morell et al, 2003) and in low amylopectin (LAPS) maize having about 90% amylose in starch (Sidebottom et al., 1998). Amylose content may be determined by size exclusion HPLC in 90% (w/v) DMSO, or by iodine blue value (iodometric method), as described in Example 1. From the grain weight and amylose content, the amount of amylose deposited per grain can be calculated and compared for transgenic and control lines.

Starch was isolated from barley grains of the T1 generation, segregating for ds-SBEIIa, or the T2 generation (probably homozygous for ds-SBEIIa) from plants transgenic for the ds-SBEIIa gene, or resulting from a cross between line IIa 4.2.5 and line IIb 4.3.8 (containing both ds-SBEIIa and ds-SBEIIb), and the amylose contents determined by the colorimetric method of Morrison and Laignelet (1983). The amylose content of starch from five pooled grain samples, listed below, was determined. The absorbance read at 650 nm was converted to percentage amylose content using the regression equation derived from standard samples (ranging from 0 to 100% amylose) made from potato amylose and amylopectin, $Y= 137.38x -30.361$, where x is the absorbance at 650nm and Y is the percentage amylose content.

Samples:

Pool 1: seven T1 seeds that showed severe starch granule distortion from the transgenic line IIa 4.1

Pool 2: six T1 seeds that showed some granule distortion from the transgenic line IIa 4.1

Pool 3: seven T1 seeds that had normal looking granules from the transgenic line IIa 4.1

Pool 4: six T2 seeds that showed severe granule distortion from the transgenic line IIa 4.2.5

Pool 5: five F1 seeds that showed severe starch granule distortion from the cross between IIa 4.2.5 and IIb 4.3.8 (ds-SBEIIb transgenic line).

Controls: Barley *SSIIa* mutant M292 (Morel et al., 2003), barley cv Himalaya and *SSIIa* wheat mutant (Yamamori et al. 2000).

Starch from grains from barley with reduced SBEIIa activity, based on the distorted starch granules, showed more than 80% amylose. The amylose content increased with the degree of distortion of the starch granules, compare pools 1, 2 and 3 (Table 6). The amylose contents for pools 1 and 2 were higher than for starch from the *SSIIa* mutant barley line M292 (Table 6). The amylose content was even higher (>90%) in the pool 5 consisting of F1 grains from the cross between the ds-SBEIIa and ds-SBEIIb transgenic lines. It is noted that the absorbance values obtained by this method may be influenced slightly by the structure of amylopectin.

Table 6. Amylose content in the grain of transgenic barley lines reduced for SBEIIa activity.

Starch sample	Amylose content (% of starch)			
	Replication 1	Replication 2	Replication 3	Mean
Pool 1	85.0	80.2	80.2	81.8
Pool 2	60.6	52.1	51.7	54.8
Pool 3	39.4	40.5	40.0	40.0
Pool 4	84.4	84.6	88.3	85.8
Pool 5	95.3	94.8	106.1	98.7
M292 barley	66.9	60.5	58.4	61.9
Himalaya barley	21.8	21.6	22.3	21.9
SSIIa wheat mutant	52.1	46.7	54.5	51.1

This implies that the amylopectin content in the starch of these grains is considerably reduced, from about 75% in wild-type to less than 20% or even less than 10%, since cereal starch is made up almost entirely of amylose and amylopectin.

EXAMPLE 7. MUTATION OF SBEIIA GENE IN BARLEY.

Mutation of the *SBEIIa* gene in barley leading to non expression of SBEIIa can be achieved through either gamma ray irradiation or chemical mutagenesis, for example with ethyl methane sulfonate (EMS). For gamma ray induced mutation, seeds are irradiated at a dose of 20-50 kR from a ^{60}Co source (Zikiryaeva and Kasimov, 1972). EMS mutagenesis is performed by treating the seeds with EMS (0.03%, v/v) as per Mullins et al., (1999). Mutant grains are identified on the basis of increased amylose content or altered starch grain morphology and confirmed by the methods described above. Mutants in *SBEIIa* can be re-mutagenized in a second round and the progeny screened for loss of SBEIIb activity in addition to SBEIIa, or the *SBEIIa* mutant can be crossed with an *SBEIIb* mutant to combine

the mutations and produce a non-transgenic variety of barley substantially lacking SBEII activity in the endosperm.

EXAMPLE 8. CLONING OF THE SBEI GENE AND CONSTRUCTS FOR INHIBITION OF SBEI EXPRESSION IN BARLEY.

Isolation of the *SBEI* gene is achieved by hybridization of probes to the barley cDNA or genomic library or by PCR methods. The PCR primer design may be based on the homologous genes from wheat, for example, based on the DNA sequence set forth in Genbank AF076679. The primers used might be 5' ACGAAGATGCTCTGCCTCAC 3' SEQ ID No. 10 and 5' GTCCAACATCATAGCCATTT 3' SEQ ID No 11 which should result in a PCR product of about 1015 bp.

The *SBEI* gene sequences are used to construct inhibitory gene constructs in a similar fashion to those described above for *SBEIIa* and *SBEIIb*, and introduced into barley.

EXAMPLE 9. COMBINATION OF *SBEIIA* MUTANTS WITH OTHER STARCH SYNTHESIS MUTANTS.

Plants transgenic for ds-SBEIIa and reduced for SBEIIa activity were crossed with the barley lines M292 (*SSIIa* mutant) and High Amylose Glacier (HAG). The following crosses were established:

1) line IIa 4.1.10 x HAG
2) line IIa 4.1.16 x HAG
3) line IIa 4.1.20 x M292
4) line IIa 4.1.19 x HAG

The F1 plants are self-fertilized and lines homozygous for both mutations are identified by genetic and molecular analysis. Combining the ds-SBEIIa transgene with the *SSIIa* mutation is expected to yield starches with very high amylose content together with high _-glucan content. Combining the ds-SBEIIa transgene with the HAG mutation may yield further alteration in starch composition with improved functionality in addition to high amylose content.

EXAMPLE 10. CHARACTERISTICS OF FIELD-GROWN BARLEY.

Kernel weights and β-glucan contents were measured for several field-grown varieties of barley including the M292 and M342 lines (*ssIIa* mutant, approx 60-65% amylose). It is noted from the results (Table 7) that M292 and M342 grain were reduced in kernel size and increased in β-glucan content relative to the wild-type varieties (3.0-6.0 % β-glucan). The average weight of field-grown wild-type grain was in the range 35-45 g/1000 kernels, grown under these conditions. The β-glucan content in the grain of wild-type varieties was in the range 3-6%.

Table 7. Kernel weight and β-glucan levels in field-grown barley.

Cultivar	1000 kernel weight [a] (g)	% beta-glucan [a]
Tantangera	34.90, 35.40	3.01, 3.37
Sloop	37.90, 41.90	3.04, 2.54
Waxiro	36.60, 37.10	5.14, 6.86
Schooner	42.60, 38.60	3.85, 3.73
Gairdner	44.80, 37.10	4.61, 4.19
Namoi	40.80, 40.80	5.19, 4.34
Himalaya	39.60, 37.90	6.04, 5.50
M292	25.10, 28.70	10.01, 9.53
M342	28.90, 30.30	8.02, 8.65
Tantangera x M292 DH	21.20, 20.40	9.08, 10.95

a: Duplicate values are given, for separate plots in the field.

It will be apparent to those skilled in the art that various modifications and alterations to these methods may be made without departing from the scope of the invention.

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CLAIMS

1. Grain obtained from a barley plant, the barley plant having a reduced level of SBEIIa enzyme activity in the endosperm, starch of said grain having a relative amylose content of at least 40% (w/w).

2. The grain of claim 1 wherein the barley plant additionally has reduced SBEIIb enzyme activity in the endosperm.

3. The grain of claim 1 wherein the barley plant comprises an exogenous nucleic acid expressing an inhibitor of SBEIIa.

4. The grain of claim 3 wherein the inhibitor causes reduced expression of the SBEIIa enzyme.

5. The grain of claim 1 wherein the grain is non-shrunken.

6. The grain of claim 5 having a starch content of at least 25% (w/w).

7. The grain of claim 6 having a starch content of at least 35% (w/w).

8. The grain of claim 7 having a starch content of about 45-50% (w/w).

9. The grain of claim 5 having an average length to thickness ratio of less than about 3.5

10. The grain of claim 5 having an average weight of at least about 36 mg.

11. The grain of claim 1 wherein the relative amylose content of the starch is at least 60% (w/w).

12. The grain of claim 11 wherein the relative amylose content of the starch is at least 70% (w/w).

13. The grain of claim 12 wherein the relative amylose content of the starch is at least 80% (w/w).

14. The grain of claim 1 which is milled, ground, pearled, rolled, kibbled, cracked or whole grain.

15. Barley grain comprising starch having a relative amylose content of at least 75% (w/w).

16. The barley grain of claim 15 wherein the amylose content is measured by the iodometric method.

17. The grain of claim 15 which comprises 3-6% (w/w) β-glucan.

18. The grain of claim 15 which comprises 6-8% (w/w) β-glucan.

19. Flour or wholemeal obtained from the grain according to any of claims 1 to 18.

20. Starch obtained from grain of a barley plant, the barley plant having a reduced level of SBEIIa enzyme activity in the endosperm, said starch being unmodified and having a relative amylose content of at least 40% (w/w).

21. The starch of claim 20 wherein the barley plant additionally has a reduced level of SBEIIb enzyme activity in the endosperm.

22. The starch of claim 20 wherein the barley plant comprises an exogenous nucleic acid expressing an inhibitor of SBEIIa.

23. The starch of claim 22 wherein the inhibitor causes a reduced level of expression of the SBEIIa enzyme.

24. The starch of claim 20 wherein the relative amylose content of the starch is at least 60% (w/w).

25. The starch of claim 24 wherein the relative amylose content of the starch is at least 70% (w/w).

26. The starch of claim 25 wherein the relative amylose content of the starch is at least 80% (w/w).

27. A composition comprising the starch according to any of claims 20 to 26 and another food ingredient or water.

28. A composition comprising starch granules of barley endosperm and another food ingredient or water, wherein the starch of the starch granules comprises at least 75% (w/w) amylose.

29. A barley plant having a reduced level of SBEIIa enzyme activity, wherein starch in grain of the barley plant has a relative amylose content of at least 40% (w/w).

30. The barley plant of claim 29 additionally having reduced SBEIIb enzyme activity in the endosperm.

31. The barley plant of claim 29 comprising an exogenous nucleic acid expressing an inhibitor of SBEIIa.

32. The barley plant of claim 31 wherein the inhibitor causes reduced expression of the SBEIIa enzyme.

33. The barley plant of claim 29 wherein the grain is non-shrunken.

34. The barley plant of claim 29 wherein the grain comprises a starch content of at least 25% (w/w).

35. The barley plant of claim 34 wherein the grain comprises a starch content of at least 35% (w/w).

36. The barley plant of claim 35 wherein the grain comprises a starch content content of about 45-50% (w/w).

37. The barley plant of claim 29 wherein the grain has an average length to thickness ratio of less than about 3.5.

38. The barley plant of claim 29 wherein the grain has an average weight of at least about 36 mg.

39. The barley plant of claim 29 wherein the relative amylose content of the starch is at least 60% (w/w).

40. The barley plant of claim 39 wherein the relative amylose content of the starch is at least 70% (w/w).

41. The barley plant of claim 40 wherein the relative amylose content of the starch is at least 80% (w/w).

42. A method of producing a barley plant capable of producing grain having starch comprising at least 40% amylose, the method comprising the steps of

a) introducing a genetic variation into a parent barley plant or seed; and

b) identifying a progeny plant or seed of the parent barley plant or seed of step a), wherein the progeny plant or seed has reduced SBEIIa activity in the endosperm.

43. The method of claim 42 wherein introducing the genetic variation of step a) comprises introducing an exogenous nucleic acid expressing an inhibitor of SBEIIa activity.

44. The method of claim 42 wherein step a) comprises mutagenesis of the parent barley plant or seed the plant is derived from.

45 A method of producing a barley plant having reduced activity of both SBEIIa and SBEIIb enzyme activities in the endosperm which comprises:

a) mutagenising seed from a plant having reduced activity of SBEIIa enzyme activity; or

b) mutagenising seed from a plant having reduced activity of SBEIIb enzyme activity; or

c) crossing a plant having reduced SEBEIIa enzyme activity with a plant having reduced SBEIIb enzyme activity;
and identifying a barley plant having reduced activity of both SBEIIa and SBEIIb.

46. Barley starch granules comprising a reduced level of SBEIIa protein and comprising starch having an amylose content of at least 40% (w/w).

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   1 GGCGAGATGG CGGAAGTAAA CATGACAGGG GGGGCTGCAG AAAAACTTGA ATCTTCAGAA
  61 CCGACTCAGG GTATTGCGGA AACAATCACT GATGGTGTAA CCAAAGGAGT TAAAGAACTA
 121 GTCGTTGGGG AGAAACCGCA AGTTGTCCCA AAACCAGGAG ATGGGCAAAA AATATACGAG
 181 ATTGACCCAA CGCTGAAAGA TTTTCGGAGC CATCTTGACT ACCGATACAG CGAATACAAG
 241 AGAATTCGTG CTGCTATTGA CCAACATGAA GGTGGATTGG AAGTTTTTTC TCGTGGTTAT
 301 GAAAAGCTTG GATTTACCCG CAGTGCTAAA GGTATCACTT ACCGAGAATG GGCTCCTGGA
 361 GCGCATTCTG CAGCATTAGT AGGTGACTTC AACAATTGGA ACCCAAATGC AGATACTATG
 421 ACCAGAGATG ATTATGGTGT TTGGGAGATT TTCCTCCCTA ACAATGCTGA TGGATCCCCT
 481 GCTATTCCTC ATGGCTCACG TGTAAAGATA CGGATGGATA CTCCATCTGG TGTGAAGGAT
 541 TCAATTTCTG CTTGGATCAA GTTCTCTGTG CAGGCTCCAG GTGAAATACC ATTCAATGGC
 601 ATATATTATG ATCCACCTGA AGAGGAGAAG TATGTCTTCC AACATCCTCA ACCTAAACGA
 661 CCAGAGTCAC TAAGGATATA TGAATCACAC ATTGGAATGA GCAGCCCGGA ACCGAAGATA
 721 AATTCATATG CTAATTTTAG GGATGAGGTG CTGCCAAGAA TTAAAAGGCT TGGATACAAT
 781 GCAGTGCAGA TAATGGCAAT CCAGGAGCAT TCATACTATG CGAGCTTTGG GTACCATGTT
 841 ACTAATTTTT TTGCACCAAG TAGCCGTTTT GGAACTCCAG AGGACTTAAA ATCCTTGATC
 901 GATAGAGCAC ATGAGCTTGG TTTGCTTGTT CTTATGGATA TTGTTCATAG TCATTCGTCA
 961 AATAATACCC TTGACGGTTT GAATGGTTTC GATGGCACTG ATACACATTA CTTCCACGGT
1021 GGTCCACGTG GCCATCATTG GATGTGGGAT TCTCGTCTGT TCAACTATGG GAGTTGGGAA
1081 GTATTAAGAT TCTTACTGTC AAACGCGAGA TGGTGGCTTG AAGAATATAA GTTTGATGGA
1141 TTTCGATTTG ATGGGGTGAC TTCCATGATG TATACTCACC ATGGATTACA AATGACATTT
1201 ACTGGGAACT ATGGCGAGTA TTTTGGATTC GCCACTGATG TTGATGCGGT GGTTTACTTA
1261 ATGCTGGTCA ACGATCTAAT TCATGGACTT TATCCGGATG CTGTATCCAT TGGTGAAGAT
1321 GTCAGCGGAA TGCCTACATT TTGCATCCCT GTCCCAGATG GTGGTGTTGG TTTTGACTAT
1381 CGCCTGCATA TGGCTGTAGC AGATAAATGG ATTGAACTCC TCAAGCAAAG TGACGAATCT
1441 TGGAAAATGG GCGATATTGT GCACACCCTA ACAAATAGAA GGTGGCTTGA GAAGTGTGTC
1501 ACTTATGCAG AAAGTCATGA TCAAGCACTA GTTGGTGACA AGACTATTGC ATTCTGGTTG
1561 ATGGATAAGG ATATGTATGA TTTCATGGCT CTGGATAGAC CTTCAACCCC TCGCATTGAT
1621 CGTGGCATAG CATTACATAA AATGATCAGG CTTGTCACCA TGGGTTTAGG TGGCGAAGGC
1681 TATCTTAATT TCATGGGAAA TGAGTTTGGG CATCCTGAAT GGATAGATTT TCCAAGAGGT
1741 CCGCAAACTC TTCCAACCGG CAAAGTTCTC CCTGGAAATA ACAATAGTTA TGATAAATGC
1801 CGCCGTAGAT TTGATCTTGG AGATGCAGAT TTTCTTAGAT ATCGTGGTAT GCAAGAGTTC
1861 GATCAGGCAA TGCAGCATCT TGAGGAAAAA TATGGGTTTA TGACATCTGA GCACCAGTAT
1921 GTTTCTCGGA AACATGAGGA AGATAAGGTG ATCATCTTCG AAAGAGGAGA TTTGGTATTT
1981 GTTTTCAACT TCCACTGGAG CAATAGCAAA AAAGACTACC GTGTTGGGTG TTCCAAGCCT
2041 GGGAAGTACA AGGTGGCCTT AGACTCTGAT GATGCACTCT TTGGTGGATT CAGCAGGCTT
2101 GATCATGATG TCGACTACTT CACAACCGAA CATCCGCATG ACAACAGGCC ACGCTCTTTC
2161 TCGGTGTACA CTCCGAGCAG AACTGCGGTC GTGTATGCCC TTACAGAGTA AGAACCAGCA
2221 GCTGTTTGTT ACAAGGCAAA AAGAGAACTC CAGTGAGCTC GTGGATTGTG AGCGAAGCGA
2281 CGGGCAACGG TCCGAGACTG TTCTAACCGC CGTGATTGGG AGGGGATCGT GCCTCTTCCC
2341 CAGATGCTAG GAGGATCAGA TGGATAGGTA GCTTGCTGGC GAGCCCTCGT TTTCAAGTGA
2401 CCTGCGAAAG AAAATGGACG GGCCTGGGTG ACATTTTGTA GTGCTGCACT GAACCATCCT
2461 ATCTCTCACA TTCCCGGTTG TTTATGTACA TATAAACTAA TAATTGCCCG TGCGCTTCAA
2521 CTTGGACAAA AAAAAAAAAA AAAAAAAAAA AAAA
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FIGURE 1

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   1 GGCGAGATGG CGGCGCCGGC GTTCGCAGTT TCCGCGGCGG GGATCGCCCG GCCATCGGCT
  61 CGTCGATCCA GCGGGGCAGA GCCGAGATCG CTGCTCTTCG GCCGCAACAA GGGCACCCGT
 121 TTCCCCCGTG CCGTCGGCGT CGGAGGTTCT GGGTGGCGCG TGGTCATGCG CGCGGGCGGC
 181 CCGTCCGGGG AGGTGATGAT CCCTGACGGC GGTAGTGGCG GAAGCGGAAC ACCGCCTTCC
 241 ATCGAGGGTT CCGTTCAGTT CGAGTCTGAT GATCTGGAGG TTCCATTCAT CGACGATGAA
 301 CCAAGCCTGC ACGATGGAGG TGAAGATACT ATTCGGTCTT CAGAGACATA TCAGGTTACT
 361 GAAGAAATTG ATGCTGAAGG CGTGAGCAGA ATGGACAAAG AATCATCCAC GGTGAAGAAA
 421 ATACGCATTG TGCCACAACC CGGAAATGGA CAGCAAATAT ACGACATTGA CCCAATGCTC
 481 CGAGACTTTA AGTACCATCT TGAGTATCGA TACAGCCTAT ATAGGAGAAT ACGTTCAGAC
 541 ATTGATGAAT ACGATGGAGG CATGGATGTA TTTTCCCGCG GCTACGAGAA GTTTGGATTT
 601 GTTCGCAGCG CTGAAGGTAT CACTTACCGA GAATGGGCTC CTGGAGCAGA TTCTGCAGCA
 661 TTAGTTGGCG ACTTCAACAA TTGGGATCCA ACTGCAGACC ATATGAGCAA AAATGACTTG
 721 GGTATTTGGG AGATTTTTCT GCCAAACAAT GCAGATGGTT CGCCGCCAAT TCCTCATGGC
 781 TCACGGGTGA AGGTGCGGAT GGATACTCCA TCTGGGACAA AGGATTCAAT TCCTGCTTGG
 841 ATCAAGTACT CCGTGCAGAC TCCAGGAGAT ATACCATACA ATGGAATATA TTATGACCCT
 901 CCTGAAGAGG AGAAGTATGT ATTCAAGCAT CCTCAACCTA AACGACCAAA ATCATTGCGG
 961 ATATATGAAA CACATGTTGG CATGAGTAGC CCGGAACCAA AGATCAACAC ATATGCAAAC
1021 TTCAGAGATG AGGTGCTTCC AAGAATTAAA AGACTTGGAT ACAATGCAGT TCAAATAATG
1081 GCAATCCAAG AGCATTCATA CTATGGAAGC TTTGGGTACC ATGTTACCAA TTTCTTTGCA
1141 CCAAGTAGCC GTTTTGGGTC CCCAGAAGAT TTAAAATCCT TGATTGATAG AGCTCACGAG
1201 CTTGGTTTGC TTGTCCTGAT GGATGTTGTT CACAGTCACG CATCAAGTAA TACCTTGGAC
1261 GGTTTGAATG GTTTTGATGG CACGGATACA CATTACTTTC ATGGCGGCTC ACGGGGCCAT
1321 CACTGGATGT GGGATTCTCG TGTGTTCAAC TACGGGAATA AGGAAGTTAT AAGGTTTCTA
1381 CTTTCCAATG CAAGATGGTG GCTAGAGGAA TATAAGTTCG ATGGTTTCCG ATTCGACGGC
1441 GCGACCTCCA TGATGTATAC CCACCATGGA TTACAAGTAA CCTTTACAGG GAGCTACCAT
1501 GAATATTTTG GCTTTGCCAC GGATGTAGAT GCAGTTGTTT ACTTGATGCT GGTGAATGAT
1561 CTAATTCACG CGCTTTATCC TGAAGCCGTT ACTATTGGTG AAGATGTTAG TGGAATGCCT
1621 ACATTTGCCC TTCCTGTTCA AGTTGGTGGG GTTGGTTTTG ACTATCGCTT ACATATGGCC
1681 GTTGCCGATA AATGGATTGA ACTTCTCAAA GGAAGCGATG AAGGTTGGGA GATGGGTAAT
1741 ATTGTGCACA CACTAACAAA TAGAAGGTGG TTGGAAAAGT GTGTTACTTA TGCTGAAAGT
1801 CATGATCAAG CACTTGTTGG AGACAAGACT ATTGCATTCT GGTTGATGGA CAAGGATATG
1861 TATGATTTCA TGGCTCTGAA CGGACCTTCG ACACCTAATA TTGATCGCGG AATAGCACTG
1921 CATAAAATGA TTAGACTTAT CACAATGGCT TTAGGAGGAG AGGGTTATCT TAACTTTATG
1981 GGAAATGAGT TCGGGCATCC TGAATGGATA GACTTTCCAA GAGGCCCACA AGTACTTCCA
2041 ACTGGTAAGT TCATCCCAGG AAATAACAAC AGTTACGACA AATGCCGTCG AAGATTTGAC
2101 CTGGGTGATG CAGAATTTCT CAGGTATCAT GGTATGCAGC AATTTGATCA GGCAATGCAG
2161 CATCTTGAGG AAAAATATGG CTTTATGACA TCAGACCACC AGTACGTATC TCGGAAACAC
2221 GAGGAAGATA AGGTGATCGT GTTTGAAAAA GGGGACTTGG TATTTGTGTT CAACTTCCAC
2281 TGGAGTAATA GCTATTTCGA CTACCGGGTC GGTTGCTTAA AGCCTGGGAA GTACAAGGTG
2341 GTGTTAGACT CAGACGCTGG ACTCTTTGGT GGATTTGGTA GGATCCATCA CACTGGAGAG
2401 CACTTCACTA ATGGCTGCCA ACATGACAAC AGGCCCCATT CGTTCTCAGT GTACACTCCT
2461 AGCAGAACCT GTGTTGTCTA TGCTCCAATG AACTAACAGC AAAGTGCAGC ATGCGCATGC
2521 GCGCTGTTGT TGCTTAGTAG CAACATAAAT CGTATGGTCA ATACAACCAG GTGCAAGGTT
2581 TAATAAGGTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTGCTTCA
2641 ACCAGTCCTG GATAGACAAG ACAACATGAT GTTGTGCTGT GTGCTCCCAA TCCCCAGGGC
2701 GTTGTGAGGA AAACATGCTC ATCTGTGTTA CCATTTTATG AATCAGCAAC GATACTTCTC
2761 CCAAAAAAAA AAAAAAAAAA
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FIGURE 2

1 AGAAACACCT CCATTTTAGA TTTTTTTTTT GTTCTTTTCG GACGGTGGGT
51 CGTGGAGAGA TTAGCGTCTA GTTTTCTTAA AAGAACAGGC CATTTAGGCC
101 CTGCTTTACA AAAGGCTCAA CCAGTCCAAA ACGTCTGCTA GGATCACCAG
151 CTGCAAAGTT AAGCGCGAGA CCACCAAAAC AGGCGCATTC GAACTGGACA
201 GACGCTCACG CAGGAGCCCA GCACCACAGG CTTGAGCCTG ACAGCGGACG
251 TGAGTGCGTG ACACATGGGG TCATCTATGG GCGTCGGAGC AAGGAAGAGA
301 GACGCACATG AACACCATGA TGATGCTATC AGGCCTGATG GAGGGAGCAA
351 CCATGCACCT TTTCCCCTCT GGAAATTCAT AGCTCACACT TTTTTTTAAT
401 GGAAGCAAGA GTTGGCAAAC ACATGCATTT TCAAACAAGG AAAATTAATT
451 CTCAAACCAC CATGACATGC AATTCTCAAA CCATGCACCG ACGAGTCCAT
501 GCGAGGTGGA AACGAAGAAC TGAAAATCAA CATCCCAGTT GTCGAGTCGA
551 GAAGAGGATG ACACTGAAAG TATGCGTATT ACGATTTCAT TTACATACAT
601 GTACAAATAC ATAATGTACC CTACAATTTG TTTTTTGGAG CAGAGTGGTG
651 TGGTCTTTTT TTTTTACACG AAAATGCCAT AGCTGGCCCG CATGCGTGCA
701 GATCGGATGA TCGGTCGGAG ACGACGGACA ATCAGACACT CACCAACTGC
751 TTTTGTCTGG GACACAATAA ATGTTTTTGT AAACAAAATA AATACTTATA
801 AACGAGGGTA CTAGAGGCCG CTAACGGCAT GGCCAGGTAA ACGCGCTCCC
851 AGCCGTTGGT TTGCGATCTC GTCCTCCCGC ACGCAGCGTC GCCTCCACCG
901 TCCGTCCGTC GCTGCCACCT CTGCTGTGCG CGCGCACGAA GGGAGGAAGA
951 ACGAACGCCG CACACACACT CACACACGGC ACACTCCCCG TGGGTCCCCT
1001 TTCCGGCTTG GCGTCTATCT CCTCTCCCCC GCCCATCCCC ATGCACTGCA
1051 CCGTACCCGC CAGCTTCCAC CCCCGCCGCA CACGTTGCTC CCCCTTCTCA
1101 TCGCTTCTCA ATTAATATCT CCATCACTCG GGTTCCGCGC TGCATTTCGG
1151 CCGGCGGGTT GAGTGAGATC TGGGCGACTG GCTGACTCAA TCACTACGCG
1201 GGGATGGCGA CGTTCGCGGT GTCCGGCGCG ACTCTCGGTG TGGCGCGGGC
1251 CGGCGTCGGA GTGGCGCGGG CCGGCTCGGA GCGGAGGGGC GGGGCGGACT
1301 TGCCGTCGCT GCTCCTCAGG AAGAAGGACT CCTCTCGTAC GCCTCGCTCT
1351 CTCGAATCTC CCCCGTCTGG CTTTGGCTCC CCTTCTCTCT CCTCTGCGCG
1401 CGCATGGCCT GTTCGATGCT GTTCCCCAAT TGATCTCCAT GAGTGAGAGA
1451 GATAGCTGGA TTAGGCGATC GCGCTTCCTG AACCTGTATT TTTTCCCCCG
1501 CGGGGAAATG CGTTAGTGTC ACCCAGGCCC TGGTGTTACC ACGGCTTTGA
1551 TCATTCCTCG TTTCATTCTG ATATATATTT TCTCATTCTT TTTCTTCCTG
1601 TTCTTGCTGT AACTGCAAGT TGTGGCGTTT TTTCACTATT GTAGTCATCC
1651 TTGCATTTTG CAGGCGCCGT CCTGAGCCGC GCGGCCTCTC CAGGGAAGGT
1701 CCTGGTGCCT GACGGCGAGA GnGACGACTT GGCAAGTCCG GCGCAACCTG
1751 AAGAATTACA GGTACACACA CTCGTGCCGG TAAATCTTCA TACAATCGTT
1801 ATTCACTTAC CAAATGCCGG ATGAAACCAA CCACGGATGC GTCAGGTTTC
1851 GAGCTTCTTC TATCAGCATT GTGCAGTACT GCACTGCCTT GTTCATTTTG
1901 TTAGCCTTGG CCCCGTGCTG GCTCTTGGGC CACTGAAAAA ATCAGATGGA
1951 TGTGCATTCT AGCAAGAACT TCACAACATA ATGCACCGTT TGGGGTTTCG
2001 TCAGTCTGCT CTACAATTGC TATTTTTCGT GCTGTAGATA CCTGAAGATA
2051 TCGAGGAGCA AACGGCGGAA GTGAACATGA CAGGGGGGAC TGCAGAGAAA
2101 CTTCAATCTT CAGAACCGAC TCAGGGCATT GTGGAAACAA TCACTGATGG
2151 TGTAACCAAA GGAGTTAAGG AACTAGTCGT GGGGGAGAAA CCGCGAGTTG
2201 TCCCAAAACC AGGAGATGGG CAGAAAATAT ACGAGATTGA CCCAACACTG
2251 AAAGATTTTC GGAGCCATCT TGACTACCGG TAATGCCTAC CCGCTGCTTT
2301 CGCTCATTTT GAATTAAGGT CCTTTCATCA TGCAAATTTG GGGAACATCA
2351 AAGAGACAAA GACTAGGGAC CACCATTTCA TACAGATCCC TTCGTGGTCT
2401 GAGAATATGC TGGGAAGTAA ATGTATAATT GATGGCTACA ATTTGCTCAA
2451 AATTGCAATA CGAATAACTG TCTCCGATCA TTACAATTAA AGAGTGGCAA
2501 ACTGATGAAA ATGTGGTGGA TGGGTTATAG ATTTTACTTT GCTAATTCCT
2551 CTACCAAATT CCTAGGGGGG AAATCTACCA GTTGGGAAAC TTAGTTTCTT
2601 ATCTTTGTGG CCTTTTTGTT TTGGGGAAAA CACATTGCTA AATTCGAATG

FIGURE 3

2651	ATTTTGGGTA	TACCTCGGTG	GATTCAACAG	ATACAGCGAA	TACAAGAGAA
2701	TTCGTGCTGC	TATTGACCAA	CATGAAGGTG	GATTGGAAGC	ATTTTCTCGT
2751	GGTTATGAAA	AGCTTGGATT	TACCCGCAGG	TAAATTTAAA	GCTTTATTAT
2801	TATGAAACGC	CTCCACTAGT	CTAATTGCAT	ATCTTATAAG	AAAATTTATA
2851	ATTCCTGTTT	TCCCCTCTCT	TTTTTCCAGT	GCTGAAGGTA	TCGTCTAATT
2901	GCATATCTTA	TAAGAAAATT	TATATTCCTG	TTTTCCCCTA	TTTTCCAGTG
2951	CTGAAGGTAT	CACTTACCGA	GAATGGGCTC	CCTGGAGCGC	ATGTTATGTT
3001	CTTTTAAGTT	CCTTAACGAG	ACACCTTCCA	ATTTATTGTT	AATGGTCACT
3051	ATTCACCAAC	TAGCTTACTG	GACTTACAAA	TTAGCTTACT	GAATACTGAC
3101	CAGTTACTAT	AAATTTATGA	TCTGGCTTTT	GCACCCTGTT	ACAGTCTGCA
3151	GCATTAGTAG	GTGACTTCAA	CAATTGGAAT	CCAAATGCAG	ATACTATGAC
3201	CAGAGTATGT	CTACAGCTTG	GCAATTTTCC	ACCTTTGCTT	CATAACTACT
3251	GATACATCTA	TTTGTATTTA	TTTAGCTGTT	TGCACATTCC	TTAAAGTTGA
3301	GCCTCAACTA	CATCATATCA	AAATGGTATA	ATTTGTCAGT	GTCTTAAGCT
3351	TCAGCCCAAA	GATTCTACTG	AATTTAGTCC	ATCTTTTTGA	GATTGAAAAT
3401	GAGTATATTA	AGGATGAATG	AATACGTGCA	ACACTCCCAT	CTGCATTATG
3451	TGTGCTTTTC	CATCTACAAT	GAGCATATTT	CCATGCTATC	AGTGAAGGTT
3501	TGCTCCTATT	GATGCAGATA	TTTGATATGG	TCTTTTCAGG	ATGATTATGG
3551	TGTTTGGGAG	ATTTTCCTCC	CTAACAACGC	TGATGGATCC	TCAGCTATTC
3601	CTCATGGCTC	ACGTGTAAAG	GTAAGCTGGC	CAATTATTTA	GTCGAGGATG
3651	TAGCATTTTC	GAACTCTGCC	TACTAAGGGT	CCCTTTTCCT	CTCTGTTTTT
3701	TAGATACGGA	TGGATACTCC	ATCCGGTGTG	AAGGATTCAA	TTTCTGCTTG
3751	GATCAAGTTC	TCTGTGCAGG	CTCCAGGTGA	AATACCTTTC	AATGGCATAT
3801	ATTATGATCC	ACCTGAAGAG	GTAAGTATCG	ATCTACATTA	CATTATTAAA
3851	TGAAATTTCC	AGTGTTACAG	TTTTTTAATA	CCCACTTCTT	ACTGACATGT
3901	GAGTCAAGAC	AATACTTTTG	AATTTGGAAG	TGACATATGC	ATTAATTCAC
3951	CTTCTAAGGG	CTAAGGGGCA	ACCAACCTTG	GTGATGTGTG	TATGCTTGTG
4001	TGTGACATAA	GATCTTATAG	CTCTTTTATG	TGTTCTCTGT	TGGTTAGGAT
4051	ATTCCATTTT	GGCCTTTTGT	GACCATTTAC	TAAGGATATT	TACATGCAAA
4101	TGCAGGAGAA	GTATGTCTTC	CAACATCTCA	ACTAAACGAC	CAGAGTCACT
4151	AAGGATTTAT	GAATCACACA	TTGGAATGAG	CAGCCCGGTA	TGTCAATAAG
4201	TTATTTCACC	TGTTTCTGGT	CTGATGGTTT	ATTCTATGGA	TTTTCTAGTT
4251	CTGTTATGTA	CTGTTAACAT	ATTACATGGT	GCATTCACTT	GACAACCTCG
4301	ATTTTATTTT	CTAATGTCTT	CATATTGGCA	AGTGCAAAAC	TTTGCTTCCT
4351	CTTTGTCTGC	TTGTTCTTTT	GTCTTCTGTA	AGATTTCCAT	TGCATTTGGA
4401	GGCAGTGGGC	ATGTGAAAGT	CATATCTATT	TTTTTTTTGT	CAGAGCATAG
4451	TTATATGAAT	TCCATTGTTG	TTGCAATAGC	TCGGTATAAT	GTAACCATGT
4501	TACTAGCTTA	AGATTTCCCA	CTTAGGATGT	AAGAAATATT	GCATTGGAGC
4551	GTCTCCAGCA	AGCCATTTCC	TACCTTATTA	ATGAGAGAGA	GACAAGGGGG
4601	GGGGGGGGGG	GGGGGTTCCC	TTCATTATTC	TGCGAGCGAT	TCAAAAACTT
4651	CCATTGTTCT	GAGGTGTACG	TACTGCAGGG	ATCTCCCATT	ATGAAGAGGA
4701	TATAGTTAAT	TCTTTGTAAC	CTACTTGGAA	ACTTGAGTCT	TGAGGCATCG
4751	CTAATATATA	CTATCATCAC	AATACTTAGA	GGATGCATCT	GAAnATTTTA
4801	GTGTGATCTT	GCACAGGAAC	CGAAGATAAA	TTCATATGCT	AATTTTAGGG
4851	ATGAGGTGTT	GCCAAGAATT	AAAAGGCTTG	GATACAATGC	AGTGCAGATA
4901	ATGGCAATCC	AGGAGCATTC	ATACTATGCA	AGCTTTGGGT	ATTCACACAA
4951	TCCATTTTTT	TCTGTATACA	CnTCTTCACC	CATTTGGAGC	TATTACATCC
5001	TAATGCTTCA	TGCACATAAA	ATATTTGGAT	ATAATCCTTT	ATTAGATATA
5051	TAGTACAACT	ACACTTAGTA	TTCTGAnnAA	nAAGATCATT	TTATTGTTGT
5101	TGGCTTGTTC	CAGGTACCAT	GTTACTAATT	TTTTTGCACC	AAGTAGCCGT
5151	TTTGGAACTC	CAGAGGACTT	AAAATCCTTG	ATCGATAGAG	CACATGAGCT
5201	TGGTTTGCTT	GTTCTTATGG	ATATTGTTCA	TAGGTAATTA	GTCCAATTTA
5251	ATTTAGCTG	TTTTACTGTT	TATCTGGTAT	TCTAAAGGGA	AATTCAGGCA
5301	ATTATGATAC	ATTGTCAAAA	GCTAAGAGTG	GCGAAAGTGA	AATGTCAAAA

FIGURE 3

5351	TCTAGAGTGG	CATAAGGAAA	ATTGGCAAAA	ACTAGAGTGG	CAAAAATAAA
5401	ATTTTCCCAT	CCTAAATGGC	AGGGCCCTAT	CGCCGAATAT	TTTTCCATTC
5451	TATATAATTG	TGCTACGTGA	CTTCTTTTTT	CTCAGATGTA	TTAAACCAGT
5501	TGGACATGAA	ATGTATTTGG	TACATGTAGT	AAACTGACAG	TTCCATAGAA
5551	TATCGTTTTG	TAATGGCAAC	ACAATTTGAT	GCCATAGATG	TGGATTGAGA
5601	AGTTCAGATG	CTATCAATAG	AATTAATCAA	CTGGCCATGT	ACTCGTGGCA
5651	CTACATATAG	TTTGCAAGTT	GGAAAACTGA	CAGCAATACC	TCACTGATAA
5701	GTGGCCAGGC	CCCACTTGCC	AGCTTCATAC	TAGATGTTAC	TTCCCTGTTG
5751	AATTCATTTG	AACATATTAC	TTAAAGTTCT	TCATTTGTCC	TAAGTCAAAC
5801	TTCTTTAAGT	TTGACCAAGT	CTATTGGAAA	ATATATCAAC	ATCTACAACA
5851	CCAAATTACT	TTGATCAGAT	TAACAATTTT	TATTTTATTA	TATTAGCACA
5901	TCTTTGATGT	TGTAGATATC	AGCACATTTT	TCTATAGACT	TGGTCAAATA
5951	TAGAGAAGTT	TGACTTAGGA	CAAATCTAGA	ACTTCAATCA	ATTTGGATCA
6001	GAGGGAACAT	CAAATAATAT	AGATAGATGT	CAACACTTCA	ACAAAAAAAT
6051	CAGACCTTGT	CACCATATAT	GCATCAGACC	ATCTGTTTGC	TTTAGCCACT
6101	TGCTTTCATA	TTTATGTGTT	TGTACCTAAT	CTACTTTTCC	TTCTACTTGG
6151	TTTGGTTGAT	TCTATTTCAG	TTGCATTGCT	TCATCAATGA	TTTTGTGTAC
6201	CCTGCAGTCA	TTCGTCAAAT	AATACCCTTG	ACGGTTTGAA	TGGTTTCGAT
6251	GGCACTGATA	CACATTACTT	CCACGGTGGT	CCACGCGGCC	ATCATTGGAT
6301	GTGGGATTCT	CGTCTATTCA	ACTATGGGAG	TTGGGAAGTA	TGTAGCTCTG
6351	ACTTCTGTCA	CCATATTTGG	CTAACTGTTC	CTGTTAATCT	GTTCTTACAC
6401	ATGTTGATAT	TCTATTCTTA	TGCAGGTATT	GAGATTCTTA	CTGTCAAACG
6451	CGAGATGGTG	GCTTGAAGAA	TATAAGTTTG	ATGGATTTCG	ATTTGATGGG
6501	GTGACCTCCA	TGATGTATAC	TCACCATGGA	TTACAAGTAA	GTCATCAAGT
6551	GGTTTCAGTA	ACTTTTTTAG	GGCACTGAAA	CAATTGCTAT	GCATCATAAC
6601	ATGTATCATG	ATCAGGACTT	GTGCTACGGA	GTCTTAGATA	GTTCCCTAGT
6651	ATGCTTGTAC	AATTTTACCT	GATGAGATCA	TGGAAGATTG	GAAGTGATTA
6701	TTATTTATTT	TCTTTCTAAG	TTTGTTTCTT	GTTCTAGATG	ACATTTACTG
6751	GGAACTATGG	CGAATATTTT	GGATTTGCTA	CTGATGTTGA	TGCGGTAGTT
6801	TACTTGATGC	TGGTCAACGA	TCTAATTCAT	GGACTTTATC	CTGATGCTGT
6851	ATCCATTGGT	GAAGATGTAA	GTGCTTACAG	TATTTATGAT	TTTTAACTAG
6901	TTAAGTAGTT	TTATTTTGGG	GATCAGTCTG	TTACACTTTT	TGTTAGGGGT
6951	AAAATCTCTC	TTTTCATAAC	AATGCTAATT	TATACCTTGT	ATGATAATGC
7001	ATCACTTAnG	TAATTTGAAA	AGTGCAAGGG	CATTCAAGCT	TACGAGCATA
7051	TTTTTTGATG	GCTGTAATTT	ATTTGATAGT	ATGCTTGTTT	GGGTTTTTCA
7101	ATAAGTGGGA	GTGTGTGACT	AATGTTGTAT	TATTTATTTA	ATTGCGGAAG
7151	AAATGGGCAA	CCTTGTCAAT	TGCTTCAGAA	GGCTAACTTT	GATTCCATAA
7201	ACGCTTTGGA	AATGAGAGGC	TATTCCCAAG	GACATGAATT	ATACTTCAGT
7251	GTGTTCTGTA	CATGTATTTG	TAATAGTGGT	TTAACTTAAA	TTCCTGCACT
7301	GCTATGGAAT	CTCACTGTAT	GTTGTnAGTG	TACACATCCA	CAAACAAGTA
7351	ATCCTGAGCT	TTCAACTCAT	GAGAAAATAn	GAnGTCCGCT	TCTGCCAGCA
7401	TTAACTGTTC	ACAGTTCTAA	TTTGTGTAAC	TGTGAAATTG	TTCAGGTCAG
7451	TGGAATGCCT	ACATTTTGCA	TCCCTGTTCC	AGATGGTGGT	GTTGGTTTTG
7501	ACTACCGCCT	GCATATGGCT	GTAGCAGATA	AATGGATTGA	ACTCCTCAAG
7551	TAAGTGCAGG	AATATTGGTG	ATTACATGCG	CACAATGATC	TAGATTACAT
7601	TTTCTAAATG	GTAAAAAGGA	AAATATGTAT	GTGAATATCT	AGACATTTGC
7651	CTGTTATCAG	CTTGAATACG	AGAAGTCAAA	TACATGATTT	AAATAGCAAA
7701	TCTCGGAAAT	GTAATGGCTA	GTGTCTTTAT	GCTGGGCAGT	GTACATTGCG
7751	CTGTAGCAGG	CCAGTCAACA	CAGTTAGCAA	TATTTTCAGA	AACAATATTA
7801	TTTATATCCG	TATATGAnGA	AAGTTAGTAT	ATAAACTGTG	GTCATTAATT
7851	GTGTTCACCT	TTTGTCCTGT	TTAAGGATGG	GCAGTAGGTA	ATAAATTTAG
7901	CCAGATAAAA	TAAATCGTTA	TTAGGTTTAC	AAAAGGAATA	TACAGGGTCA
7951	TGTAGCATAT	CTAGTTGTAA	TTAATGAAAA	GGCTGACAAA	AGGCTCGGTA
8001	AAAAAAACTT	TATGATGATC	CAGATAGATA	TGCAGGAACG	CGACTAAAGC

FIGURE 3

8051	TCAAATACTT	ATTGCTACTA	CACAGCTGCC	AATCTGTCAT	GATCTGTGTT
8101	CTGCTTTGTG	CTATTTAGAT	TTAAATACTA	ACTCGATACA	TTGGCAATAA
8151	TAAACTTAAC	TATTCAACCA	ATTTGGTGGA	TACCAGAnAT	TTCTGCCCTC
8201	TTGTTAGTAA	TGATGTGCTC	CCTGCTGCTG	TTCTCTGCCG	TTACAAAAGC
8251	TGTTTTCAGT	TTTTTGCATC	ATTATTTTTG	TGTGTGAGTA	GTTTAAGCAT
8301	GTTTTTTGAA	GCTGTGAGCT	GTTGGTACTT	AATACATTCT	TGGAAGTGTC
8351	CAAATATGCT	GCAGTGTAAT	TTAGCATTTC	TTTAACACAG	GCAAAGTGAC
8401	GAATCTTGGA	AAATGGGCGA	TATTGTGCAC	ACCCTAACAA	ATAGAAGGTG
8451	GCTTGAGAAG	TGTGTAACTT	ATGCAGAAAG	TCATGATCAA	GCACTAGTTG
8501	GTGACAAGAC	TATTGCATTC	TGGTTGATGG	ATAAGGTACT	AGCTGTTACT
8551	TTTGGACAAA	AGAATTACTC	CCTCCCGTTC	CTAAATATAA	GTCTTTGTAG
8601	AGATTCCACT	ATGGACCACA	TAGTATATAG	ATGCATTTTA	GAGTGTAGAT
8651	TCACTCATTT	TGCTTCGTAT	GTAGTCCATA	GTGAAATCTC	TACAGAGACT
8701	TATATTTAGG	AACGGAGGGA	GTACATAATT	GATTTGTCTC	ATCAGATTGC
8751	TAGTGTTTTC	TTGTGATAAA	GATTGGCTGC	CTCACCCATC	ACCAGCTATT
8801	TCCCAACTGT	TACTTGAGCA	GAATTTGCTG	AAAACGTACC	ATGTGGTACT
8851	GTGGCGGCTT	GTGAACTTTG	ACAGTTATGT	TGCAATTTTC	TGTTCTTATT
8901	TATTTGATTG	CTTATGTTAC	CGTTCATTTG	CTCATTCCTT	TCCGAGACCA
8951	GCCAAAGTCA	CGTGTTAGCT	GTGTGATCTG	TTATCTGAAT	CTTGAGCAAA
9001	TTTTATTAAT	AGGCTAAAAT	CCAACGAATT	ATTTGCTTGA	ATTTAAATAT
9051	ACAGACGTAT	AGTCACCTGG	CTCTTTCTTA	GATGATTACC	ATAGTGCCTG
9101	AAGGCTGAAA	TAGTTTTGGT	GTTTCTTGGA	TGCCGCCTAA	AGGAGTGATT
9151	TTTATTGGAT	AGATTCCTGG	CCGAGTCTTC	GTTACAACAT	AACATTTTGG
9201	AGATATGCTT	AGTAACAGCT	CTGGGAAGTT	TGGTCACAAG	TCTGCATCTA
9251	CACGCTCCTT	GAGGTTTTAT	TATGGCGCCA	TCTTTGTAAC	TAGTGGCACC
9301	TGTAAGGAAA	CACATTCAAA	AGGAAACGGT	CACATCATTC	TAATCAGGAC
9351	CACCATACTA	AGAGCAAGAT	TCTGTTCCAA	TTTTATGAGT	TTTTGGGACT
9401	CCAAAGGGAA	CAAAAGTGTC	TCATATTGTG	CTTATAACTA	CAGTTGTTTT
9451	TATACCAGTG	TAGTTTTATT	CCAGGACAGT	TGATACTTGG	TACTGTGCTG
9501	TAAATTATTT	ATCCGACATA	GAACAGCATG	AACATATCAA	GCTCTCTTTG
9551	TGCAGGATAT	GTATGATTTC	ATGGCTCTGG	ATAGGCTTCA	ACTCTTCGCA
9601	TTGATCGTGG	CATAGCATTA	CATAAAATGA	TCAGGCTTGT	CACCATGGGT
9651	TTAGGTGGTG	AAGGCTATCT	TAACTTCATG	GGAAATGAGT	TTGGGCATCC
9701	TGGTCAGTCT	TTACAACATT	ATTGCATTCT	GCATGATTGT	GATTTACTGT
9751	AATTTGAACC	ATGCTTTTCT	TTCACATTGT	ATGTATTATG	TAATCTGTTG
9801	CTTCCAAGGA	GGAAGTTAAC	TTCTATTTAC	TTGGCAGAAT	GGATAGATTT
9851	TCCAAGAGGC	CCACAAACTC	TTCCAACCGG	CAAAGTTCTc	CCCTGGAAAT
9901	AACAATAGTT	ATGATAAATG	CCGCCGTAGA	TTTGATCTTG	TAAGTTTTAG
9951	CTGTGCTATT	ACATTCCCTC	ACTAGATCTT	TATTGGCCAT	TTATTTCTTG
10001	ATGAAATCAT	AATGTTTGTT	AGGAAAGATC	AACATTGCTT	TTGTAGTTTT
10051	GTAGACGTTA	ACATAAGTAT	GTGTTGAGAG	TTGTTGATCA	TTAAAAATAT
10101	CATGATTTTT	TGCAGGGAGA	TGCAGATTTT	CTTAGATATC	GTGGTATGCA
10151	AGAGTTCGAT	CAGGCAATGC	AGCATCTTGA	GGAAAAATAT	GGGGTATGTC
10201	ACTGGTTTGT	CTTTGTTGCA	TAACAAGTCA	CAGTTTAACG	TCAGTCTCTT
10251	CAAGTGGTAA	AAAAAGTGTA	GAATTAATTC	CTGTAATGAG	ATGAAAACTG
10301	TGCAAAGGCG	GAGCTGGAAT	TGCTTTTCAC	CAAAACTATT	TTCTTAAGTG
10351	CTTGTGTATT	GATACATATA	CCAGCACTGA	CAATGTAACT	GCAGTTTATG
10401	ACATCTGAGC	ACCAGTATGT	TTCACGGAAA	CATGAGGAAG	ATAAGGTGAT
10451	CATCCTCnAA	AAGAGGAGAT	TTGGTATTTG	TTTTCAACTT	CCACTGGAGC
10501	AATAGCTTTT	TTGACTACCG	TGTTGGGTGT	TCCAAGCCTG	GGAAGTACAA
10551	GGTATGCTTG	CCTTTTCATT	GTCCACCCTT	CACCAGTAGG	GTTAGTGGGG
10601	GCTTCTACAA	CTTTTAATTC	CACATGGATA	GAGTTTGTTG	GTCGTGCAGC
10651	TATCAATATA	AAGAATAGGG	TAATTTGTAA	AGAAAAGAAT	TTGCTCGAGC
10701	TGTTGTAGCC	ATAGGAAGGT	TGTTCTTAAC	AGCCCCGAAG	CACATACCAT

FIGURE 3

```
10751   TCATTCATAT tATCTACTTA AGTGTTTGTT TCAATCTTTA TGCTCAGTTG
10801   GACTCGGTCT AATACTAGAA CTATTTTCCG AATCTACCCT AACCATCCTA
10851   GCAGTTTTAG AGCAGCCCCA TTTGGACAAT TGGCTGGGTT TTTGTTAGTT
10901   GTGACAGTTT CTGCTATTTC TTAATCAGGT GGCCTTGGAC TCTGACGATG
10951   CACTCTTTGG TGGATTCAGC AGGCTTGATC ATGATGTCGA CTACTTCACA
11001   ACCGTAAGTC TGGGCTCAAG CGTCACTTGA CTCGTCTTGA CTCAACTGCT
11051   TACAAATCTG AATCAACTTC CCAATTGCTG ATGCCCTTGC AGGAACATCC
11101   GCATGACAAC AGGCCGCGCT CTTTCTCGGT GTACACTCCG AGCAGAACTG
11151   CGGTCGTGTA TGCCCTTACA GAGTAAGAAC CAGCAGCGGC TTGTTACAAG
11201   GCAAAGAGAG AACTCCAGAG AGCTCGTGGA TCGTGAGCGA AGCGACGGGC
11251   AACGGCGCGA GGCTGCTCCA AGCGCCATGA CTGGGAGGGG ATCGTGCCTC
11301   TTCCCCAGAT GCCAGGAGGA GCAGATGGAT AGGTAGCTTG TTGGTGAGCG
11351   CTCGAAAGAA AATGGACGGG CCTGGGTGTT TGTTGTGCTG CACTGAACCC
11401   TCCTCCTATC TTGCACATTC CCGGTTGTTT TTGTACATAT AACTAATAAT
11451   TGCCCGTGCG CTCAACGTGA AAATC
```

FIGURE 3

```
   1 AAGCTTTGTA GCCTTGCACG GGCTCCCCAA CAAACTGCCT CACTCGATTG
  51 TCAAAAAAGT AAAAATGATT GTAGAAAAAA AAACTGACTC ACTCGTCACT
 101 ACCCTACCGT CCTACATGAC ACCTGGCCGC AAGACGACGC CGTCCTCCTG
 151 CCGCGCGCGT CCGCGATCAC ACCACCGCAA AAACCAAAAC CTCTTCGCCG
 201 GTGCGTCCCA CGCTACCATC CATGCAGCCG TCCGCCCGCG CGCGCGTTGC
 251 CCGCACCACC CGCTGGCGGC CACCACGCCG CCACTCTCGC GTGAAGGCTC
 301 CGTCCGCTTC CTCCTAGTTC CACTCTCTCT CCGTGCTAGC AGTATATAGC
 351 ATCCGCCCTC CGCCCCCTCC CAATCTTAGA ACACCCCTCC CTTTGCCTCC
 401 TCATTTCGCT CGCGTGGGTT TAAGCAGGAG ACGAGGCGGG GTCAGTTGGG
 451 CAGTTAGGTT GGATCCGATC CGGCTGCGGC GGCGGCGACG GGATGGCTGC
 501 GCCGGCATTC GCAGTTTCCG CGGCGGGGCT GGCCCGGCCG TCGGCTCCTC
 551 GATCCGGCGG GGCAGAGCGG AGGGGGCGCG GGGTGGAGCT GCAGTCGCCA
 601 TCGCTGCTCT TCGGCCGCAA CAAGGGCACC CGTTCACCCC GTAATTATTT
 651 GCGCCACCTT TCTCACTCAC ATTCTCTCGT GTATTCTGTC GTGCTCGCCC
 701 TTCGCCGACG ACGCGTGCCG ATTCCGTATC GGGCTGCGGT GTTCAGCGAT
 751 CTTACGTCGG TTCCCTCCTG GTGTGGTGAT GTCTGTAGGT GCCGTCGGCG
 801 TCGGAGGTTC TGGATGGCGC GTGGTCATGC GCGCGGGGGG GCCGTCCGGG
 851 GAGGTGATGA TCCCTGACGG CGGTAGTGGC GGAACACCGC CTTCCATCGA
 901 CGGTCCCGTT CAGTTCGATT CTGATGATCT GAAGGTAGTT TTTTTTTTGC
 951 ATCGATCTGA AGGTACTTGA CATATACTAC TGTATTACCC TGAGTAAATA
1001 CTGCCACCAT ATTTTTATGG TTCGCTTGAA ATACCTGTTT ACTTGCTACG
1051 GTTTTCACTT TCATTGAGAC GTCGGACGAA ATTCACTGAA TTCCTATAAT
1101 TTGGTAGACA CCGAAATATA TACTACTCCT TCCGTCCCAT AATATAAGAG
1151 CGTTTTTGGC ACCTTATATT ATAGGGCGGA GGGAGTACCT TTTAGGTCAA
1201 AATATTGTGG TAGTTTCAAT TGTATACAAG AATTCAAATA TTTTTTTTAA
1251 AAAAAAATCA ACTAATTGGT TGAGTTTCAA GTGAAGCGTT TTGGTCCTTT
1301 GGCTGAGATG TAAACCGAAA TCACTGAAAT TCATAGTAGC CGAAACTTTA
1351 ATAGAACTGA AACTCAAAAT CTGCTATCCG GCGAAATTCT AAAGATTTGC
1401 TTATTTCACA CGTAGGTTGC AGTACACCCT CTTTCTAATT TATTGGGGAA
1451 GGGGTATTAT TATCTTGTTA GTACCTGCCT GCATGACAAT TGAAATCTAA
1501 GACAAAACAC CATATGCGAG GCCTACACAC GGTAGGTTGG TTTACAACTA
1551 TGTGTGCCAC AGTTCGTCTG AACTTTTTGT CCTTCACATC GTGTTAGGTT
1601 CCATTCATTG ATGATGAAAC AAGCCTACAG GATGGAGGTG AAGATAGTAT
1651 TTGGTCTTCA GAGACAAATC AGGTTAGTGA AGAAATTGAT GCTGAAGACA
1701 CGAGCAGAAT GGACAAAGAA TCATCTACGA GGGAGAAATT ACGCATTCTG
1751 CCACCACCGG GAAATGGACA GCAAATATAC GAGATTGACC CAACGCTCCG
1801 AGACTTTAAG TACCATCTTG AGTATCGGTA TGCTTCGCTT CTATTGTGTG
1851 CACTTTAAAA ACAATTTACA GTCTTTGATA AGATGTGAAT GGCTGCTTGC
1901 TGTGACACGA AACTCTTGAA GTTCGTAGTC ACTCTTGTGT GTTCATGGTT
1951 CTGAGGTAAC ATGGTAACCG AACAAAAATA GGAAAGTGGC AAGCACTGCA
2001 ATGTGAGCTA CTGATAACCA CCCATTGTAA TTGGGTACAC TGATTAATAT
2051 ATATGTCTTC ATGGGCTCTA TTTTTTTTCA ATATCTATGC CAATTGAACA
2101 ACAATGCTTT GTGGACGGGT GTTCTTTTAC CCTCTTCTTC TATCAATAGA
2151 TGATATGCAT ACTCATGCGT ATCCTACAAA AAATTGAACA ACAATGCCAC
2201 TTTCCCCCGT GTTGCTTTTG TAAGGATGAA ACACATATGT CCAGATCAAA
2251 CTATACTAGC AGTCTAACTG TGCCTTAATG GATCAAAAAC AGATATAGCC
2301 TATACAGGAG AATACGTTCA GACATTGATG AACACGAAGG AGGCATGGAT
2351 GTATTTTCCC GCGGTTACGA GAAGTTTGGA TTTATGCGCA GGTGAAATTT
2401 CTTGACTAAA TAACTATGTA TCTACCTTTT CTTTGTACTC TATCAACATT
2451 CCTCTTCCCA TGCAGCGCTG AAGGTATCAC TTACCGAGAA TGGGCTCCTG
2501 GAGCAGATGT ACGTTCTTCT AACCATCTGA TCGTTTACCT GACTATACTA
2551 ATTCTATCTT TCAACTAATT GTGAATAATT ACTGCTCATC AGCTATCCTA
2601 AGGTTGGGGA TTTTGCACCT CCCAGATGAA CAGCATATTA AGTCGCACAA
2651 CTAGCATTAT TAAGAACTAA CTCCTGCTTC CAATTGCAGT CTGCAGCATT
2701 AGTTGGCGAC TTCAACAATT GGGATCCAAA TGCAGACCAT ATGAGCAAAG
2751 TATGCATGTA GTTTCACAAA TATATCATAT TTTCTTTGTA GATTTTTTTT
```

FIGURE 4

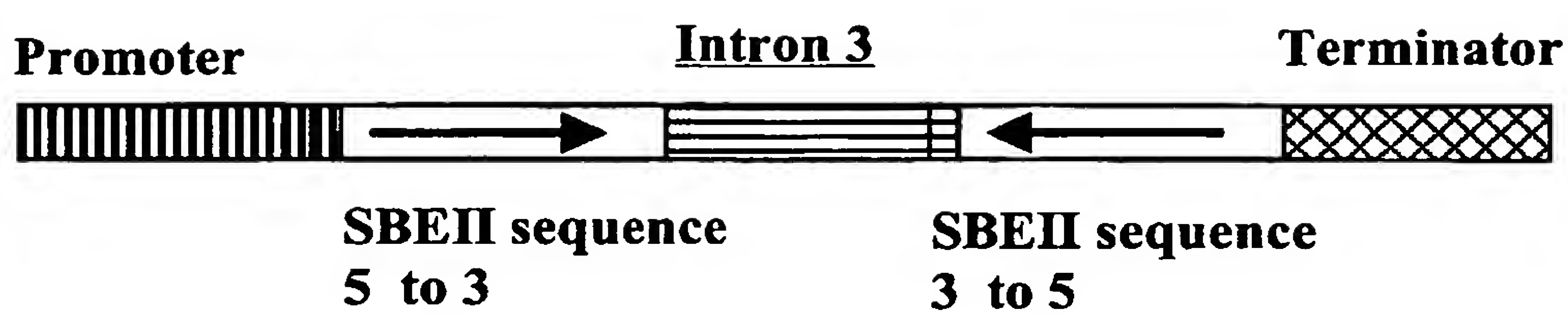
```
2801  TTTAGATCGG CTTATCTATT TAAATGTGGT TGAATATACA CCTTATATGT
2851  ACGTTGAGCT GTAAATATAG TTGGAAGTGT TTAGGAGTAT TAAATTCACT
2901  GGACTCTATT CTTTCACTTG CCTGTTGCAC GAGCCCATTA CTAGATATCA
2951  ATGTTGATGA TGCTTTTGTT GTATGAGGTC GAAGTGAAAC ATGCATGTTA
3001  CCCTTTTATA TAAGTAAGGT TGCACATGTA TTTTTATGA TCTAAACATT
3051  ATTTACTGAT TTTGTTCTTG CAAGACACTA AGCAGTTTTA CATAATAATG
3101  GCGTTGGAGC AGGCCGACTG CACATCTGAA CTGTAGCTCC ATGTGGTTGA
3151  TATAGATTAC AAATGCTCAT ATTCAATGTA ACTGTTTTCA GAATGACCTT
3201  GGTGTTTGGG AGATTTTTCT GCCAAACAAT GCAGATGGTT CGCCACCAAT
3251  TCCTCACGGC TCACGGGTGA AGGTTGTTTT CTTCTCCTTG CCAACGGTGT
3301  TAGGCTCAGG AACATGTCCT GTATTACTCA GAAGCTCTTT TGAACATCTA
3351  GGTGAGAATG GATACTCCAT CTGGGATAAA GGATTCAATT CCTGCTTGGA
3401  TCAAGTACTC CGTGCAGACT CCAGGAGATA TACCATACAA TGGAATATAT
3451  TATGATCCTC CCGAAGAGGT ATTTTACTTC ATCTTCTGTG CTTTTAGATT
3501  TCAGATATTT TTATTAGAAG AAAATTATGA TTTTTTCCCT CACGAACCTT
3551  CCCAATTGCT ATTTCAAGCT GTCCTACTTA TTTGCTGCTG GCATCTTATT
3601  TTTCTATTCT CTAACCAGTT ATGAAATTCC TTACATGCAT ATGCAGGAGA
3651  AGTATGTATT CAAGCATCCT CAACCTAAAC GACCAAAATC ATTGCGGATA
3701  TATGAAACAC ATGTTGGCAT GAGTAGCCCG GTATTTCATC TTTACCATGT
3751  ATTCCATAAA TGAAGTTAGC TATATGCAGT TCAAATTTAT TTACAGGTTG
3801  TTACAATGGT ATTTTTGTGT TGGTGCCCTT CTTTCGTTTT ATAAGTAAAA
3851  AACTTATCAT AAATTTATTT GTTATGCCGC TTGGTTAATA CAATCTGAAA
3901  AATGTAACTG TGGACAATCT AGAACTAGAT AATACAAATC TGAAAAAACA
3951  TGCTGGAATA GTGTCATTTC AGTCAACTAG GATGTTTTGA ATGCTCAAGA
4001  GAAGTACTAG TGTGTAGCAT CAAAAGCTGG TGTCCATTTG TTCAAATGTT
4051  TAATTAACAC TATAGTGAAA ACAAGTAATT GCACAAAGAA ACAAGTAATT
4101  GCCCAAGTTC ATATGTTTTT TCACTATATT ACATGTTTCA TCAACAATTT
4151  AATTAACCTC ATTCCTTACA AACATTTGTA TTTACATTTG TTCCTACATA
4201  TATAGTTATT TTATATATCA ACTTTATAAA TCATGACTGT TATAATTAAA
4251  ACCGATGGTA TATCAACGAT TGAGATAATT TGGCATATGT GGATGAATTT
4301  TGTGGCTTGT TATGCTCTTG TTTTAATAAC ATAATAAATA GATTATGCTT
4351  GTTGGTAGCC TTTTTACATT AACACATGGG CAATTACTTG TTTCTTTGTG
4401  CAACCAGGAA CCAAAGATCG ACACATATGC AAACTTCAGG GATGAGGTGC
4451  TTCCAAGAAT TAAAAGACTT GGATACAATG CAGTGCAAAT AATGGCAATC
4501  CAAGAGCACT CATACTATGG AAGCTTTGGG TAGTTCTCTG GGTCGATTTC
4551  TGGTTCTTTT AGTTATCTTT TGTCCATAGA ACATATTTCA ACTTTAGCAA
4601  CTATACTATT ATATTAACTT TTCAGCTATT GTCTTNCTTT TTCTTATGTG
4651  AGAGACTGCT GCNTCTTGCT ACTTCCTGTG TTCTCATTCA GAGTANACAT
4701  CTTATGANTA GACAACTCTA TGTNGACATT CCGGAAGTAT NCACTGGCTG
4751  ATTCGGTCTA AAATAACATA CTGCTCAGAT AGCCACATAA CAGTACGATT
4801  ACACACATAA TGACCATGTT TGCATAGAGT GGCGGTAGTA TGTTCCTCAC
4851  CATACTAGCA TAATGACTTG TTATATAAGA GTATATCATA TTAACTTCTT
4901  TTCCAATGAC ATGGAAGCTG TAACAACTTT CAAATCATTT TTGTCTTTTA
4951  AGTGCTGCTT TTTTCCTGTT TGACAATTAA TACAATACCA CTTTTATGTG
5001  TTTTTACTTC TATTGCAGGT ACCATGTTAC CAATTTCTTT GCACCAAGTA
5051  GCCGTTTTGG GTCCCCAGAA GATTTAAAAT CTTTGATTGA TAGAGCTCAC
5101  GAGCTTGGCT TGGTTGTCCT CATGGATGTT GTTCACAGGT ACTTAATGTA
5151  ATTTGAGGTT GGCGTGTTAA GTTCACATTA ATCTTAATTC TTTATTTCAA
5201  TTCCTATGGC CTCTCTCCTA GATTGGAACA GTAAAAGCAT CATCCAGTTT
5251  GTATAAATTG CTAAAAGAAC ATTTTACATG TTAAGTATTT TCAATTACTA
5301  TGAAACATAT AAATTTACAT ACTTATTGAT TTTACGACAG AAGTACCGAT
5351  CTCACAAGAT GAACAATTGG TTGATCACAT ATCATTTCAT ACTACAATAC
5401  AAGAAAATGA ATAGAGAACG AGTTAATATT AGCCTTGGTA AAATCAGCAA
5451  CTTGTTTGGA AATAAAGTAT AGTGATGCCA GTGCAAANAA CAAGGCATCA
5501  AGTTGGTTTC AGCTCCCACG GTCGGTGCTA GCTGTCAAGG GTAATTTGCA
5551  CGTAGTCGCA CATAGATTTG TGTGGGAGTG GAAAGTAACC ACAGATTGTC
```

FIGURE 4

5601	CGAGGAACAC	GGGACACACG	TCTTAGCCAC	AGGTTTGGGC	TCCCCTTGAT
5651	GCGGGTAGTA	GCTTTACTCC	TTATATGAAA	TTATCTCAAG	ATAGATTTCA
5701	ATTTGGGGTT	ACACTTANGA	ACTCANCAAG	TTAAGGATCA	ACTCNCTGAG
5751	TTCTATACGA	CTGATCTTTG	ACCGAGATAT	CTTGATCAGG	CTAAGTANCA
5801	AAATCCAGGC	CTTGAGATGT	TGAACATGTC	CTTCATTTTG	GGCTGGGTGC
5851	CCTTGGGCAT	AAGGTGTNGT	CCTTCCTTCA	TGTGCTTCTT	GCAGCGTATG
5901	ACATAAACNT	CCTCTGAGTT	GGTANATGCA	CGGTTCCCTT	TGAGGAAATC
5951	AGGGGTAGTC	GCATCTNGGG	AAAGTTGGTC	ACCCANGCAT	GGATCCTCNG
6001	CGCACACCGG	GCAAACACGG	TGAAACCACT	TCTCCTCGAC	ACTAGCTAAC
6051	TTGACATTCA	AGCAAACTAA	GAATATAACT	TTATNTCTAA	ATGAACCGGA
6101	CACCCTCCTT	GTGCCTGCAC	CTACAGAGTA	CAATGCCAGT	TTTGGACTGA
6151	ACTCTTGTGT	TCATGTATGT	GCTAATNACA	TAGGTTCTAA	CCATGATTCT
6201	AAATAGCGCG	TTATAACTCC	ACTATAGTAA	TGCTATAGCG	TTTANAAGAT
6251	CCCGCACTAA	GGGACCTTAG	TCCAAATACA	TGATCAAACA	TTTTACATAG
6301	CGCGCTATAG	CTATTTAAAA	CTATGGTCAC	CCGCTAAGAG	GCATAACTCG
6351	CTATTTAAAA	CTATGGTTCT	AACTTTTAAT	CTATTTTATG	TCTTGGTCCA
6401	AAGCCCCTTT	TTGTTCTATA	GCTTTACCTT	TGGGTTGAGA	TCACCCTTAA
6451	CCCATTGGTA	ATCCTGGTTG	ATTTACTCCA	TCCTTTCTTG	CGTAGCTTTA
6501	CTTTTGGTTT	TTTGTTTCTC	ACAGTCACGC	GTCAAATAAT	ACCTTGGACG

FIGURE 4

A


Promoter
Intron 3
Terminator
SBEII sequence
5 to 3
SBEII sequence
3 to 5

B

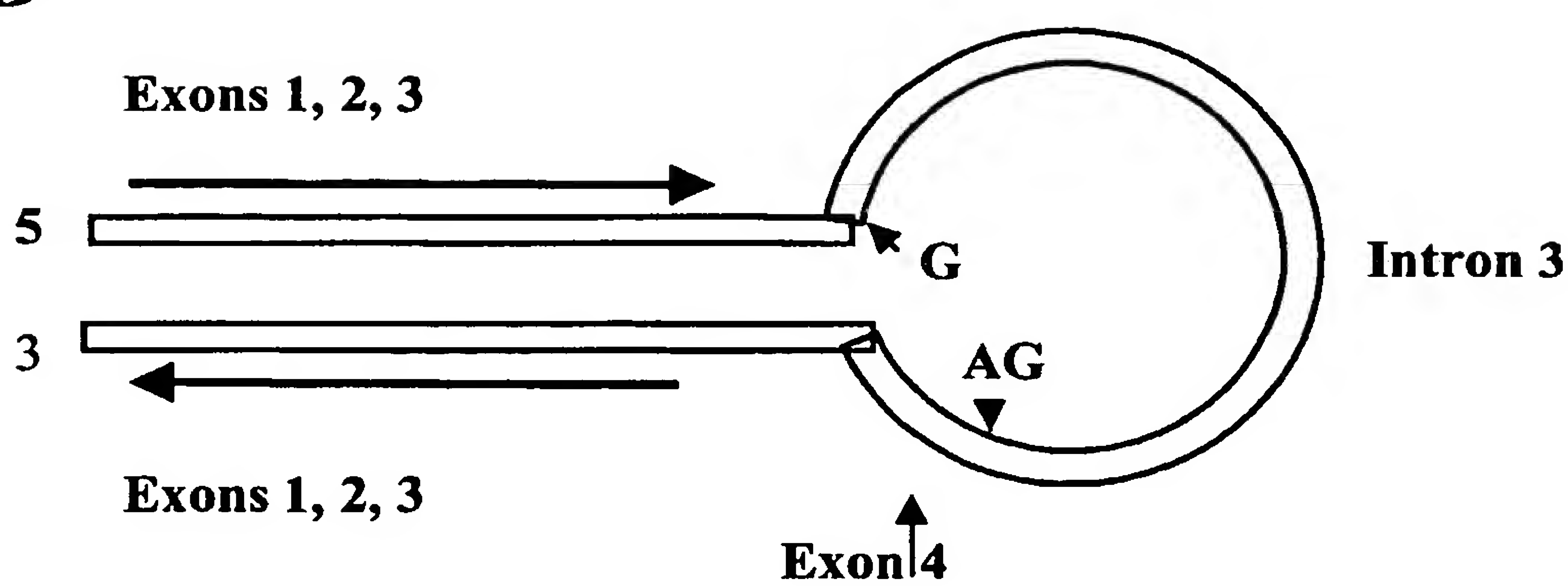

Exons 1, 2, 3
5
3
G
AG
Intron 3
Exons 1, 2, 3
Exon 4

FIGURE 5

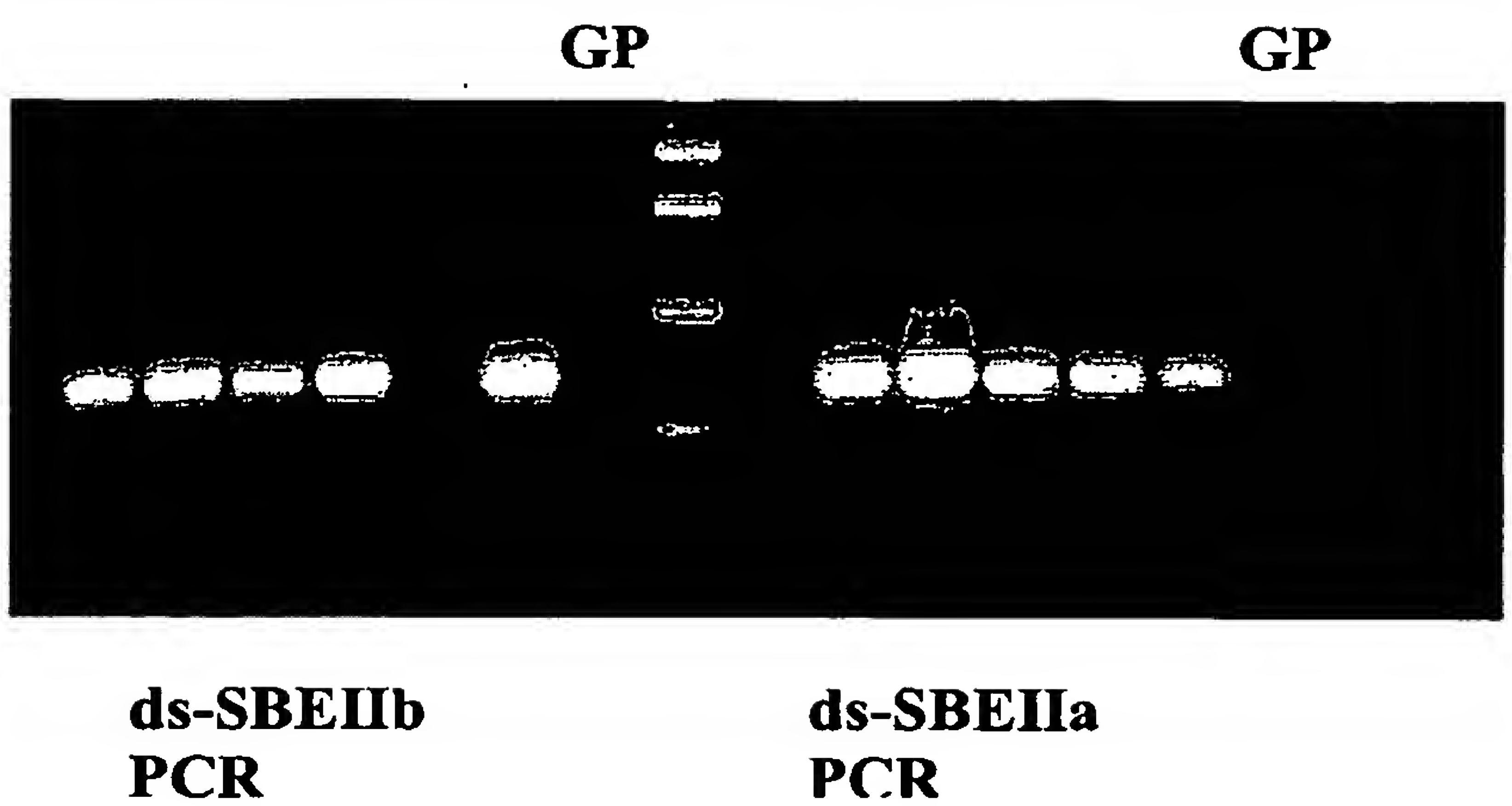
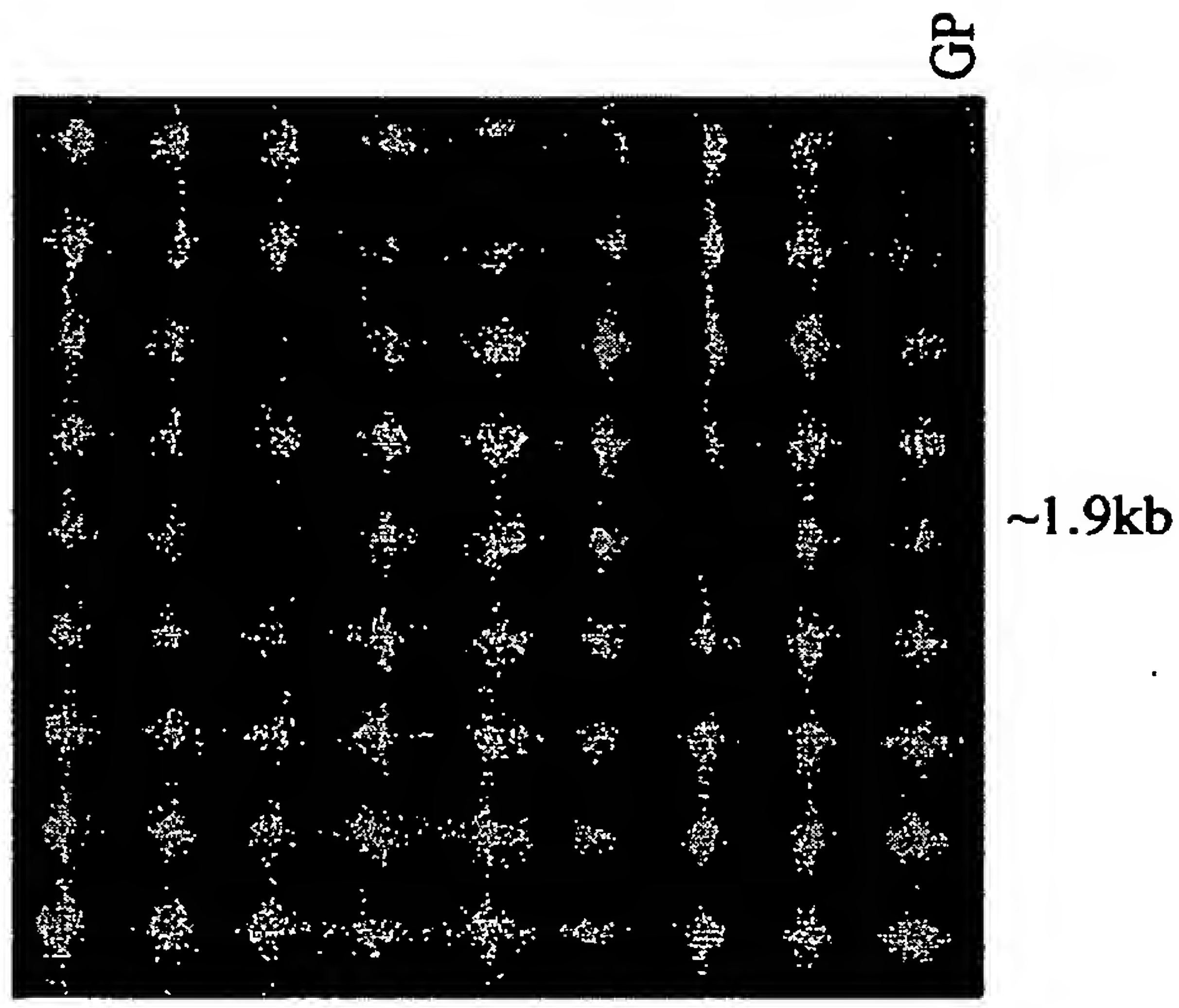


FIGURE 6

A



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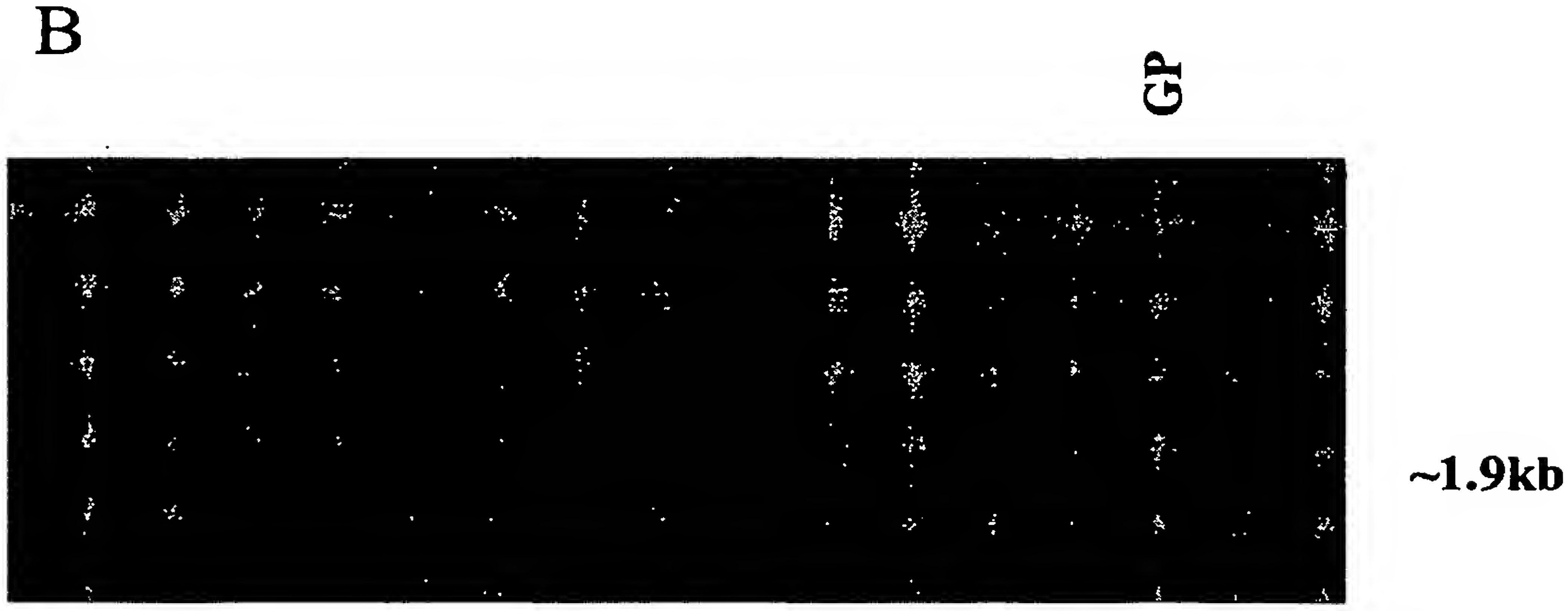


FIGURE 7

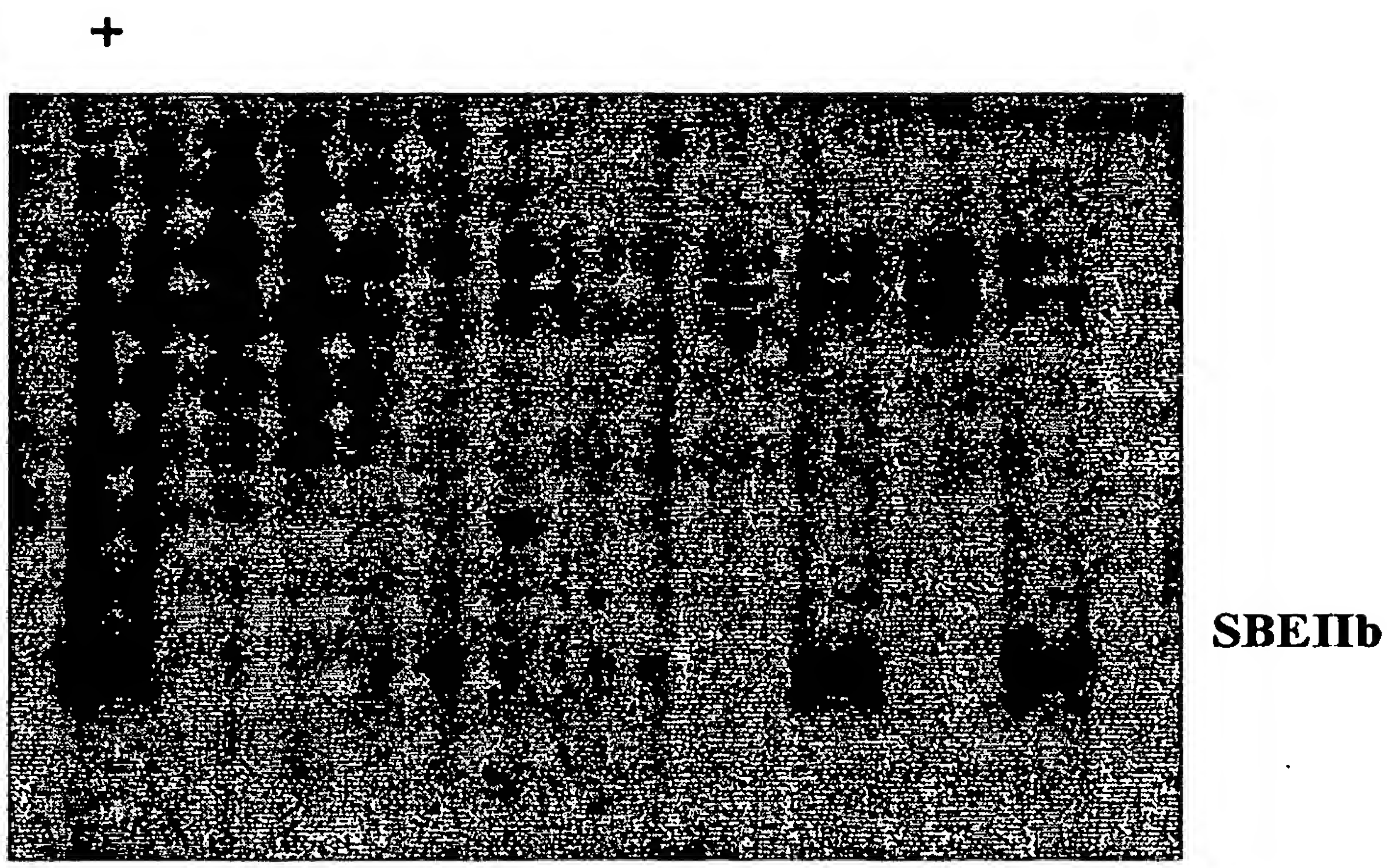


FIGURE 8

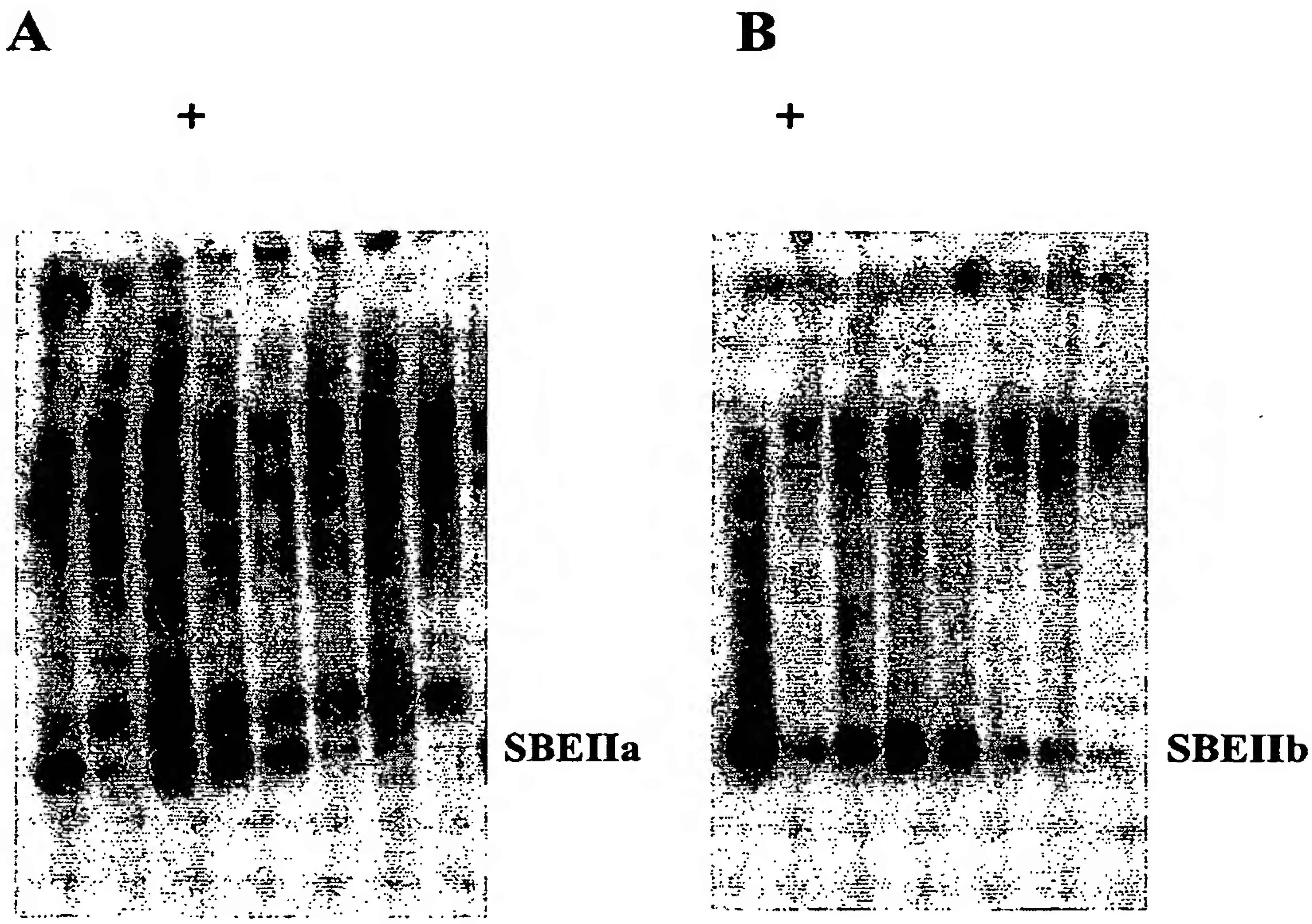


FIGURE 9

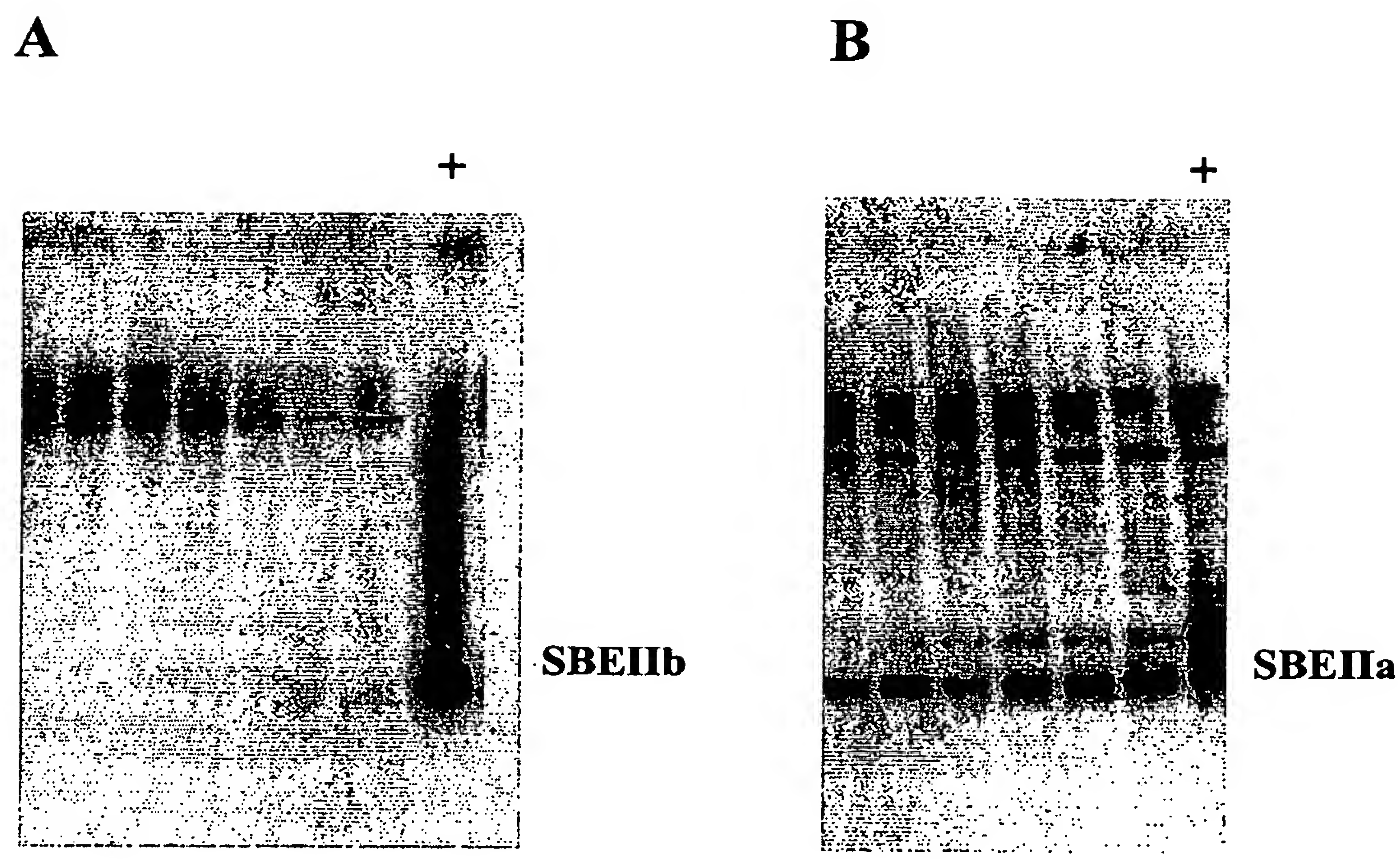
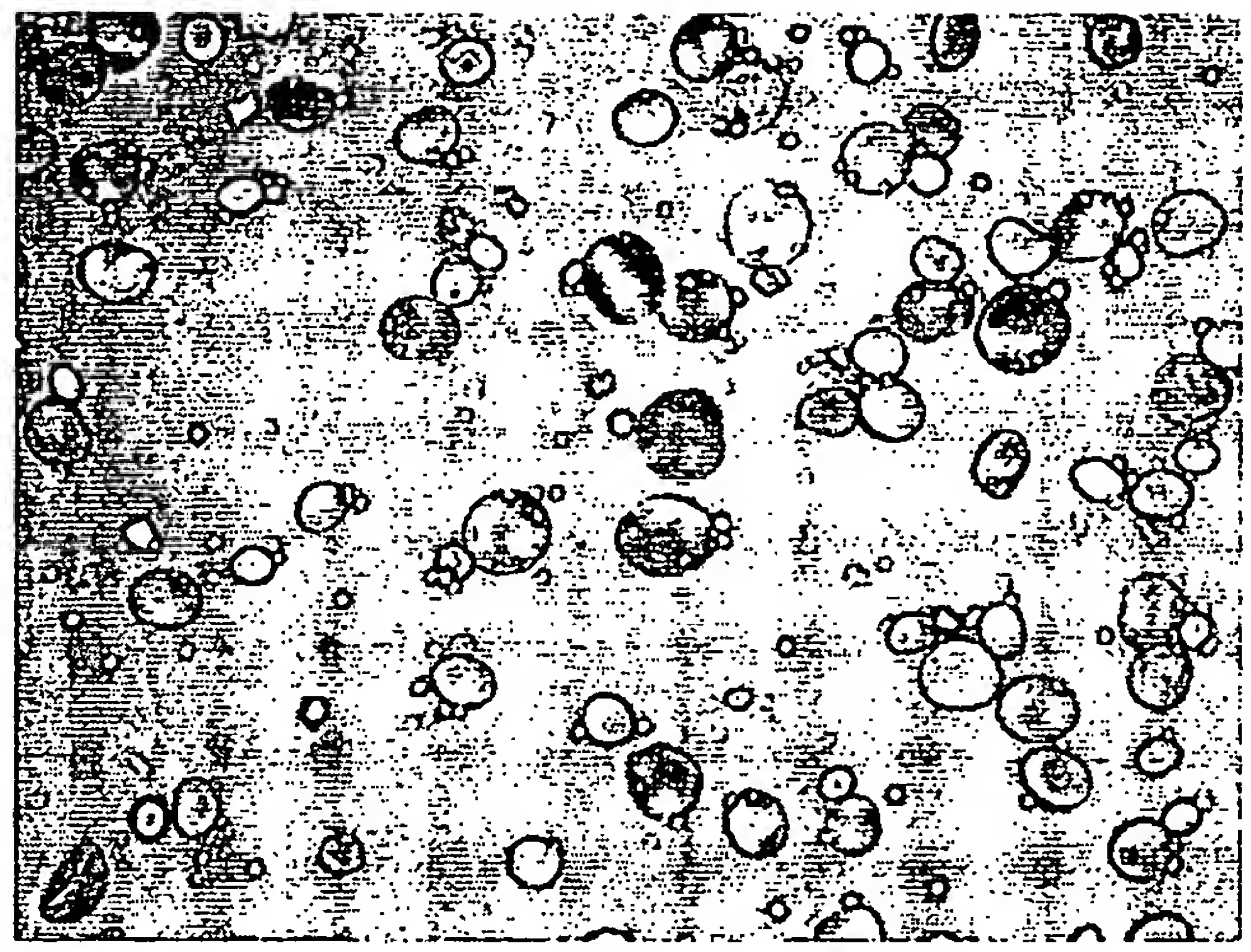


FIGURE 10

A



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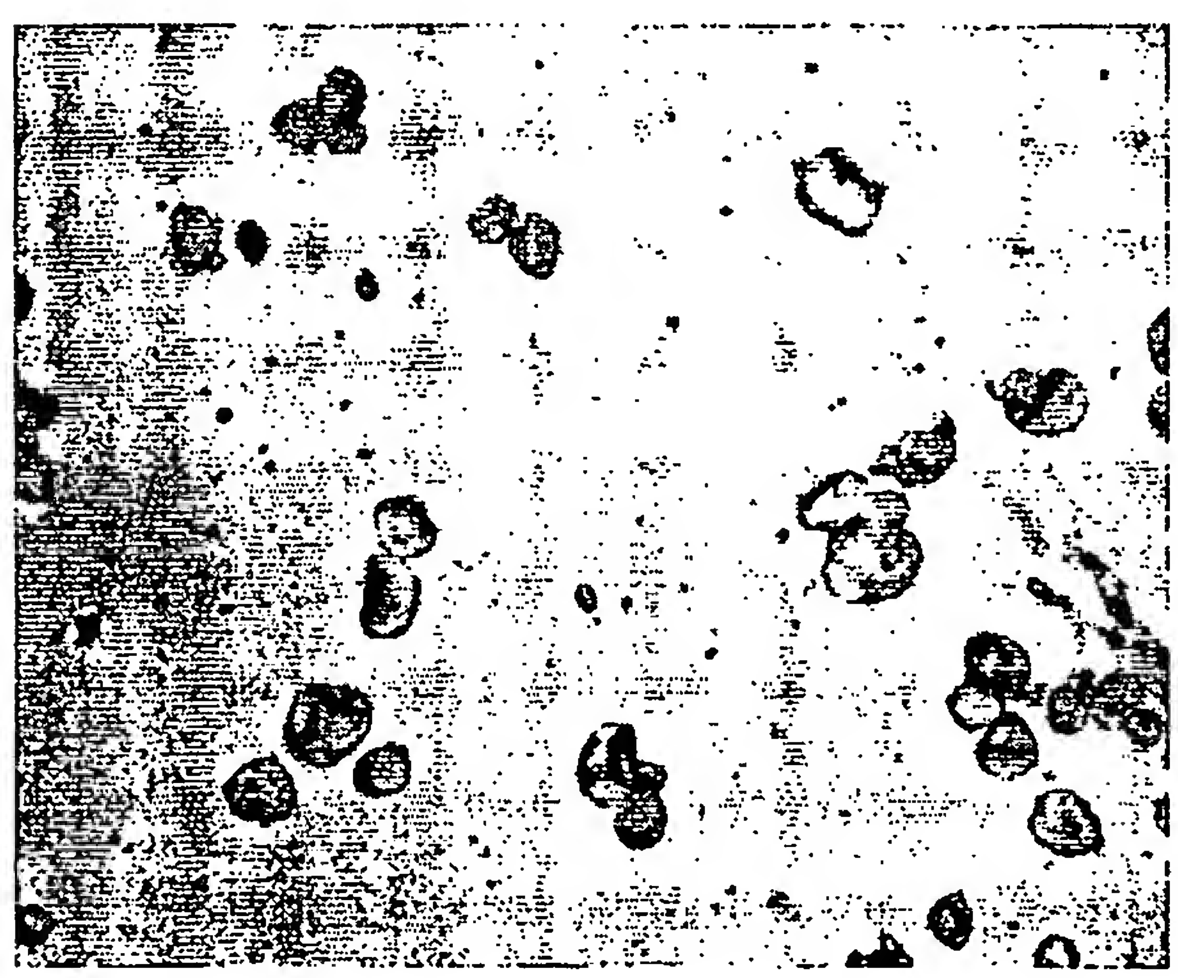

FIGURE 11

A

B

C

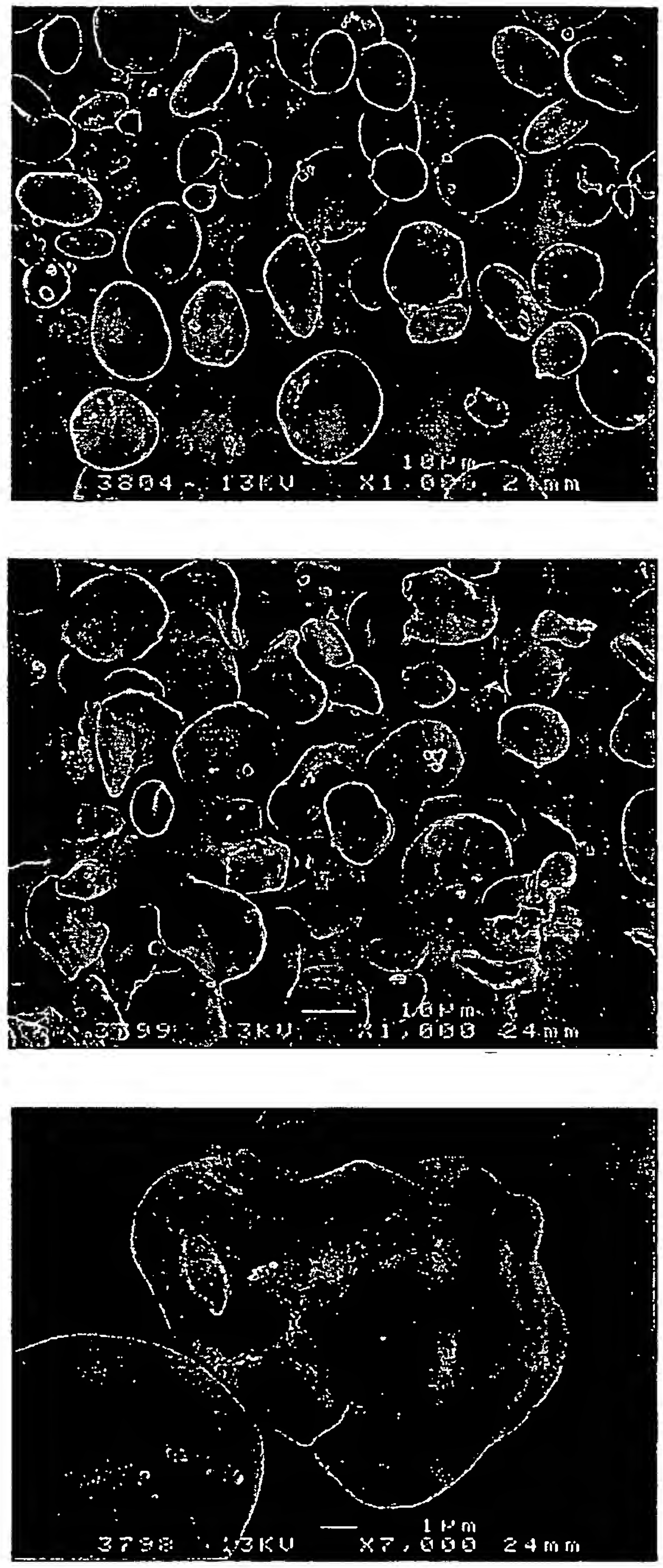

FIGURE 12

Sequence Listing

<110> Commonwealth Scientific and Industrial Research Organisation
<120> Barley with altered branching enzyme activity and starch and starch containing products with an increased amylose content
<160> 11
<210> 1
<211> 2554
<212> DNA
<213> Hordeum vulgare
<220>
<223> SSBEIIa cDNA
<400> 1

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gtcgttgggg agaaaccgca agttgtccca aaaccaggag atgggcaaaa aatatacgag     180
attgacccaa cgctgaaaga ttttcggagc catcttgact accgatacag cgaatacaag     240
agaattcgtg ctgctattga ccaacatgaa ggtggattgg aagtttttc tcgtggttat      300
gaaaagcttg gatttacccg cagtgctaaa ggtatcactt accgagaatg ggctcctgga     360
gcgcattctg cagcattagt aggtgacttc aacaattgga acccaaatgc agatactatg     420
accagagatg attatggtgt ttgggagatt ttcctcccta acaatgctga tggatcccct     480
gctattcctc atggctcacg tgtaaagata cggatggata ctccatctgg tgtgaaggat     540
tcaatttctg cttggatcaa gttctctgtg caggctccag gtgaaatacc attcaatggc     600
atatattatg atccacctga agaggagaag tatgtcttcc aacatcctca acctaaacga     660
ccagagtcac taaggatata tgaatcacac attggaatga gcagcccgga accgaagata     720
aattcatatg ctaattttag ggatgaggtg ctgccaagaa ttaaaaggct tggatacaat     780
gcagtgcaga taatggcaat ccaggagcat tcatactatg cgagctttgg gtaccatgtt     840
actaattttt ttgcaccaag tagccgtttt ggaactccag aggacttaaa atccttgatc     900
gatagagcac atgagcttgg tttgcttgtt cttatggata ttgttcatag tcattcgtca     960
aataataccc ttgacggttt gaatggtttc gatggcactg atacacatta cttccacggt    1020
ggtccacgtg gccatcattg gatgtgggat tctcgtctgt tcaactatgg gagttgggaa    1080
gtattaagat tcttactgtc aaacgcgaga tggtggcttg aagaatataa gtttgatgga    1140
tttcgatttg atggggtgac ttccatgatg tatactcacc atggattaca aatgacattt    1200
actgggaact atggcgagta ttttggattc gccactgatg ttgatgcggt ggtttactta    1260
atgctggtca acgatctaat tcatggactt tatccggatg ctgtatccat tggtgaagat    1320
gtcagcggaa tgcctacatt ttgcatccct gtcccagatg gtggtgttgg ttttgactat    1380
cgcctgcata tggctgtagc agataaatgg attgaactcc tcaagcaaag tgacgaatct    1440
tggaaaatgg gcgatattgt gcacacccta acaaatagaa ggtggcttga gaagtgtgtc    1500
acttatgcag aaagtcatga tcaagcacta gttggtgaca agactattgc attctggttg    1560
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cgtggcatag cattacataa aatgatcagg cttgtcacca tgggtttagg tggcgaaggc    1680
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cgccgtagat ttgatcttgg agatgcagat ttccttagat atcgtggtat gcaagagttc    1860
gatcaggcaa tgcagcatct tgaggaaaaa tatgggttta tgacatctga gcaccagtat    1920
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00565

A. CLASSIFICATION OF SUBJECT MATTER	
Int. Cl. [7]: A01H 5/00 C12N 15/29	
According to International Patent Classification (IPC) or to both national classification and IPC	
B. FIELDS SEARCHED	
Minimum documentation searched (classification system followed by classification symbols) CA, WPIDS	
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched SEE ELECTRONIC DATABASES	
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) MEDLINE, CAPLUS, AGRICOLA, WPIDS (starch, amylopectin, branching enzyme, Q enzyme, 1,4-alpha glucan branching enzyme, SBEII?, SBE2?, muta?, inhibit?, antisense, RNAi, dsRNA, reduc?, plant, barley, hordeum, monocot?, wheat, triticum, EC 2.4.1.18)	

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P/X	WO0237955 A (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 16 May 2002. (See p.25 Line 22 - p.26 Line 29; p.31 Lines 20-29; p.37 Line 5 - p.39 Line 28)	15-19, 28
X	WO0015810 A (PLANT BREEDING INTERNATIONAL CAMBRIDGE LIMITED) 23 March 2000. (See p.4 Line 23 - p.14 Line 13; Seq ID 15 and 16; Examples)	1-46
X	Jansson, C. *et al.* 1997. Cloning, characterization and modification of genes encoding starch branching enzymes in barley. In: P.J. Frazier *et al* (eds), Starch Structure and Functionality. The Royal Society of Chemistry, Cambridge, UK. pp 196-203. (See whole document).	1-46

[X] Further documents are listed in the continuation of Box C   [X] See patent family annex

* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search 19 June 2003	Date of mailing of the international search report 26 JUN 2003
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **DAVID OLDE** Telephone No : (02) 6283 2569

C (Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Sathish, P. *et al.* 1995. Cloning and anti-sense RNA constructs of a starch branching enzyme gene from barley endosperm. In: P. Mathis (ed.), Photosynthesis: from Light to Biosphere. Kluwer Academic Publishers, Netherlands. Vol 5:313-316. (See whole document).	15-19, 28
A	WO0162934 A (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 30 August 2001. (See whole document).	1-46
A	Blauth, S.L. *et al.* 2001. Identification of *Mutator* insertional mutants of starch-branching enzyme 2a in corn. Plant Physiology. 125:1396-1405. (See whole document).	1-46
A	GB2360521 A (DANISCO A/S) 26 September 2001. (See whole document).	1-46
A	Sun, C. *et al.* 1998. The two genes encoding starch-branching enzymes IIa and IIb are differentially expressed in barley. Plant Physiology. 118:37-49. (See whole document).	1-46
A	WO9722703 A (E.I. DU PONT DE NEMOURS AND COMPANY) 26 June 1997. (See whole document).	1-46
A	Sun, C. *et al.* 1997. Identification of four starch-branching enzymes in barley endosperm: partial purification of forms I, IIa and IIb. New Phytologist. 137: 215-222. (See whole document).	1-46
A	Schondelmaier, J. *et al.* 1992. Genetical studies on the mode of inheritance and localization of the *amo1* (High amylose) gene in barley. Plant Breeding. 109:274-280. (See whole document).	1-46

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU03/00565

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	200237955	AU	20001370	AU	200214804	AU	20001371
		AU	20001372	AU	20001373		
WO	200015810	AU	58725/99	CZ	20010759	EP	1117814
		HU	200103618	PL	346568		
WO	200162934	AU	20005742	AU	200135237	CA	2400710
		EP	1263961				
GB	2360521	AU	200139506	CA	2402463	EP	1265477
		WO	200170942				
WO	9722703	AU	16846/97	BR	9612086	CA	2239979
		CN	1219199	EP	868520	HU	9902112
		US	6376749	ZA	9610590		

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